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13. ABSTRACT (Maximum 200 Words)
It is hypothesized hat the short term objectives of doing this proposal are to better understand which sex steroids and which cellular immune functions control post resection metastatic cancer spread. The long term goal is to use this understanding to develop testable hypotheses for "neoadjuvant" hormonal/immunological therapies, which when given at or around the time of a cancer resection, might result in diminishing the subsequent metastatic spread of the resected cancer. Progress has been made in each of the three technical directives and nine tasks, as outlined in the annual report.

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Surgery, neoplasm, estrous cycle, cellular immunity, chronobiology

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Introduction:

We have found that the timing of breast cancer resection within the mouse's and the women's fertility cycle determine whether or not that operation cures the mouse or woman or whether the cancer spreads throughout the hosts. This project proposed to determine the effect of removal of the ovaries on this biology. It also proposed to determine whether and how cellular immunity is altered differentially by resection at specific phase within the cycle. The ultimate goal is to use the understanding generated to develop a peri-surgical therapy to increase the chances of curing this disease. We have done some progression toward this goal.

Body:

Technical Objective #1: Establish tumors, followed by surgical resection of tumors in mice at each of four estrous cycles and/or *in vivo* hormonal modulations, followed by determination of metastatic recurrence. We proposed five tasks "Task #1-5" in this objective.

Task 1- "Resect tumors in intact mice at one of four fertility cycle stages with no hormone modulation; follow up tumor recurrence with lung metastasis". This task has been accomplished in our previous and current laboratory. The following abstract summarizes our findings.

Fertility cycle influence on surgical breast cancer cure.

Cancer growth and spread is an intricate process dependent upon both tumor and host. This laboratory is interested in the role of the fertility cycle, specifically cyclic changes in steroid hormone levels, in tumor growth and metastases. Our previous studies, using a murine model, have documented that breast cancer growth rate and post-resection metastatic behavior each change reproducibly during the estrous cycle, and that post-resection cancer spread depends upon the time within the estrous cycle that an advanced transplanted cancer is resected. Twelve to thirty-two percent cure rates were seen in these studies. That early work described estrous cycle stages just prior and near to putative ovulation to be superior while those stages farther from ovulation were disadvantageous times for surgery. Data presented here confirm the role of the estrous cycle in post-resection metastatic spread. This current work validates vaginal smear determined estrous cycle stage with uterine weight. A primary, transplantable, mammary carcinoma, which metastasizes to the lungs, was resected for surgical cure in cycling C3HeB/FeJ female mice at each fertility cycle stage. A group of oophorectomized (ovx) animals was also used. In two large, independent studies resecting much earlier stage cancers than in prior studies, a 96% surgical cure frequency was documented when the tumor is resected during estrus. The second best surgical cure rate is achieved when tumors are resected

during metestrus (79% overall cure rate). Cure frequency in ovx animals is intermediate. These results further support a probable role for circulating E2 and P4 levels in modulating the metastatic process. We conclude that the timing of surgical resection within the estrous cycle affects the cancer's metastatic potential and that the optimal timing of resection may also depend to some extent upon the size (stage) of the resected cancer. (Please see the attached file#1 for details. Bove K, Lincoln DW, Wood PA, Hrushesky WJ. Fertility cycle influence on surgical breast cancer cure. Breast Cancer Res Treat. 2002 75:65-72.)

Task 2- Resect tumors in OVX mice with no hormone modulation. This task has also been accomplished in our previous and current laboratory as mentioned in above abstract.

Taks 3, 4- Ablate hormones (estrogen, progesterone) in ***intact and ovx*** mice with tumors using pellets with ICI 182780, RU486 -> Resect tumors -> Follow up tumor recurrence with lung metastasis. We have done some preliminary in vivo experiments on this task and ongoing data analysis. Recently, we have established an in vitro tumor cell line from the original tumor line in our laboratory. Using this in vitro cell model, we have conducted experiments in which the effects of sex hormonal exposure on tumor cell DNA synthesis and proliferation were studied (please see following abstracts).

(1). Creation of a Stable Breast Cancer Cell Line That Maintains Fertility Cycle Cancer Biology of the Parent Tumor

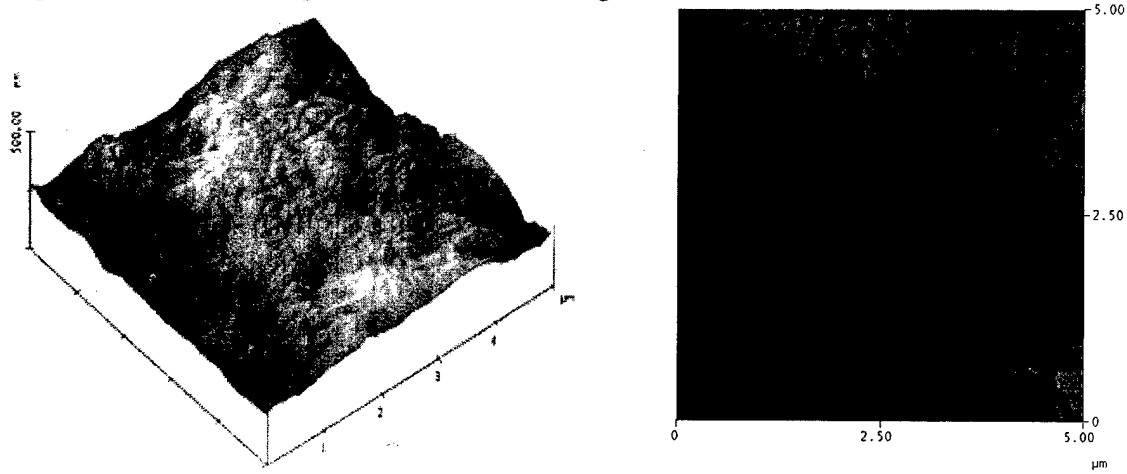
A mammary tumor cell line, designated MTCL, was successfully established from a mouse primary mammary tumor (MTP). The MTCL cells retain both estrogen receptors (ER) and progesterone receptors (PR) *in vitro*. *In vitro* exposure of MTCL cells to progesterone causes a decrease in the cellular ^{3}H-thymidine uptake, indicating an inhibition by progesterone on MTCL cellular DNA synthesis; while exposure of the cells to a high dose of estrogen (15 pg/ml) for 48 hours causes an increase of ^{3}H-thymidine uptake. We inoculated both MTP or MTCL tumor cells into normal cycling female C_3HeB/FeJ mice and demonstrated that the post resection metastatic recurrence of MTCL tumors, like the original MTP tumors, depends upon the time of tumor resection within the mouse estrous cycle stage. We demonstrated that both MTCL and MTP tumors have similar histological appearances but with significant differences in tumor necrosis and vascularity. Equivalent levels of sex hormonal receptors (ER α, ER β, and PR), epithelial growth hormonal receptors (Her2/neu, EGFR1), tumor suppressors (BRCA-1, P53), and cell apoptosis relevant protein (bcl-xl) were found in these *in vivo* tumors, except that the cyclin E protein was significantly higher in MTP tumor cells comparing to that in MTCL tumor cells. Our results indicate that MTCL cells retain many of biological features of the original MTP primary tumor cells, and to our knowledge, it is the first *in vitro* cell line that has been shown to maintain the unique estrous cycle dependence of *in vivo* cancer metastasis. (Please see the attached file#2 for details. Shaojin You, Wei Li, Minoru Kobayashi, Yin Xiong, William Hrushesky, Patricia Wood. Creation of a Stable Breast Cancer Cell Line That Maintains Fertility Cycle Cancer Biology of the Parent Tumor. This paper has been submitted for publish)

(2). Tumor cell surface topography and estrous cycle relevant hormonal milieu

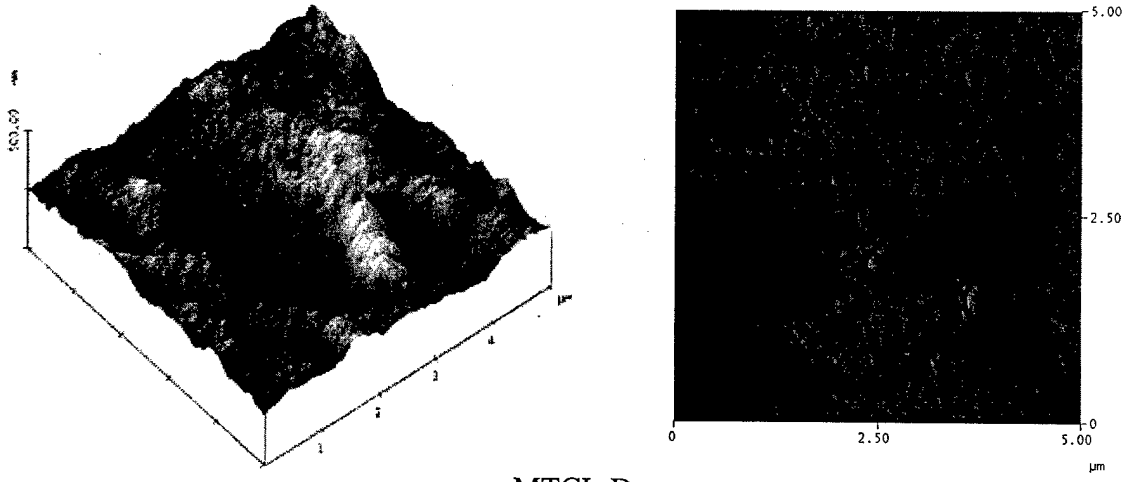
The atomic force microscope (AFM), one of the scanning probe microscopes, is a useful tool to examine the surface structure on cultured tumor cells. AFM is expected to give the images of structures such as cell surface receptors, channels and carbohydrates chains. Some of these molecules are the key players in tumor cell metastatic spread.

Understanding how the estrous cycle relevant hormonal milieus consequentially change the tumor cell surface structures is of fundamental importance to elucidate the mechanisms by which the sex hormones modulate tumor metastasis potential. To gain new insights into these cell surface structural studies, we recently cultured and exposed MTCL cells to combinations of progesterone and estrogen that mimic four estrous cycle relevant sex hormonal milieus (P, E, M and D). Analysis of the Atomic Force Microscope (AFM) images (Figure 1, Table 1) of the MTCL cells reveals significant variations in different hormonal exposures. Tumor cells exposed to D, M and P (MTCL-D, MTCL-M, and MTCL-P) show relatively higher roughness than that exposed to MTCL-E. The MTCL-D and MTCL-P especially show significantly higher overall height variations than the control.

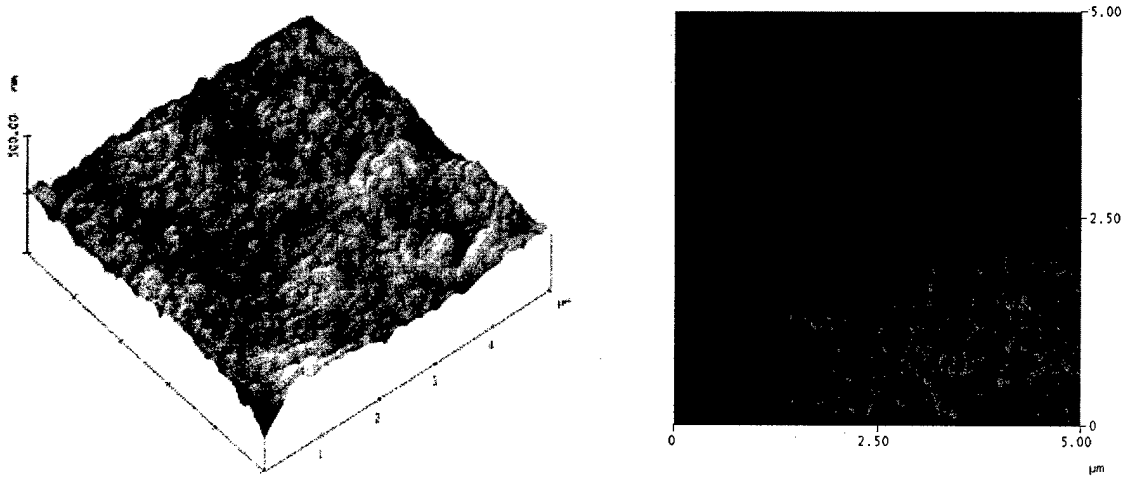
Figure 1. AFM 3D height and 2D friction images of MTCL tumor cells.



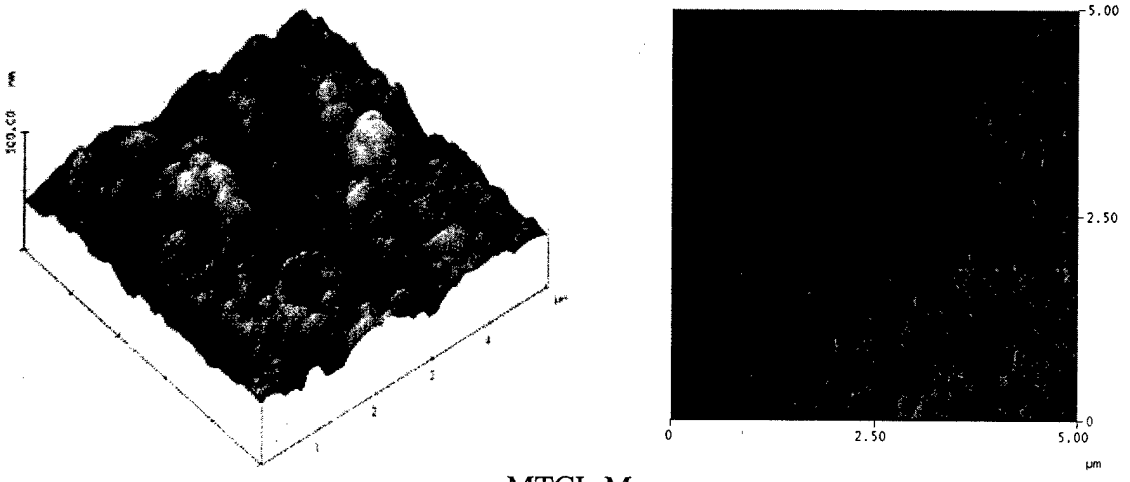
MTCL-con



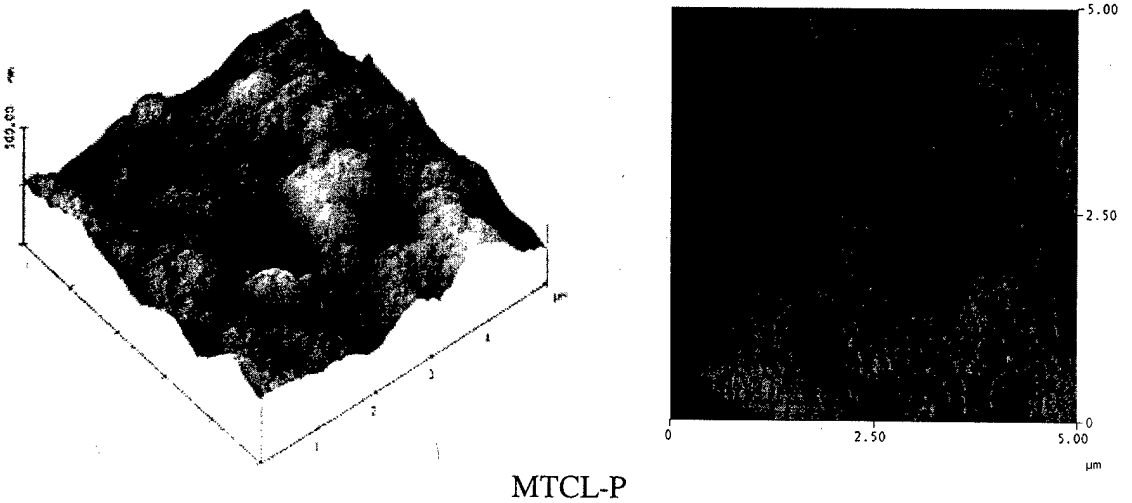
MTCL-D



MTCL-E



MTCL-M



MTCL-P

Table 1. Roughness and grain-size data for cell samples.

Sample	RMS Roughness (nm)	Height Range (nm)	Avg. Grain Size (nm)
MTCL	16.06	142.86	251.78
MTCL-D	28.84	184.96	158.58
MTCL-E	13.63	145.88	256.15
MTCL-M	24.66	163.02	381.01
MTCL-P	35.57	244.17	164.64

(3). Establish a stably GFP transfected MTCL cell line to study cancer chronobiology

GFP tagged tumor cell model provides a powerful tool for tumor metastatic studies. The GFP-expressing tumor cells can emit bright fluorescent light and the light signal is strong enough so that the external images of GFP-expressing tumors can be obtained in free-moving mice with help of whole-body imaging technique, namely a fluorescent microscope and video camera, without any staining procedure needed. This technique will be especially helpful in studying the early stage of tumor metastasis. We have lately succeeded in transferring a GFP expressing gene vector into MTCL tumor cells. In this experiment, we seeded 5x 10^5 MTCL cells in each 60 mm culture dishes and cultured the cells until 70% confluence, and then transfected them with the pEGFP-IRIS-hyg plasmid (Clontech,Palo Alto, CA) using the TransFast^{TM} Transfection reagent (Promega). We conducted series of experiments to optimize efficacy of the transfection in order to achieve the maximum GFP transfection rate. To stabilize the GFP transfected MTCL tumor cells, we replaced the regular culture medium with the selective culture medium, which is contained 200μg/ml hygromycin (BD Biosciences-clontech). Once all of the untransfected tumor cells were eliminated, we expended the GFP transfected tumor cells into new dishes with regular culture medium. The percentage of the green transfected cells was evaluated under fluorescent microscope before the cells were passed into new dishes. Currently, we have already passed this stabilized GFP tumor cell line over 10 generations *in vitro* and the percentage of the green tumor cells still kept at about 95% (**figure 2**).

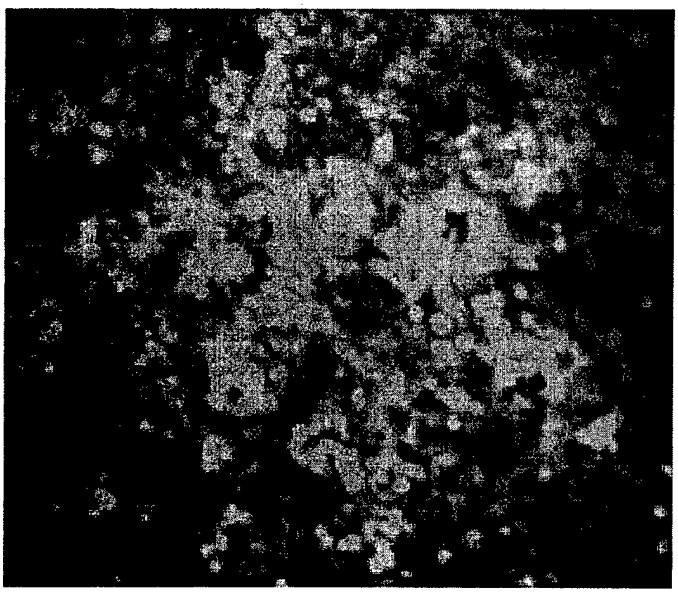


Figure 2.

To test how long the GFP expression would last *in vivo*, we implanted them on the back of female C3HeB/FeJ mice s.c. By following up the intensity of GFP fluorescence in the tumor cells, we have demonstrated that the expression of GFP in the MTCL tumor cells can last about two months *in vivo* (three *in vivo* passages from mouse to mouse). In another preliminary experiment, we injected 5x 10^5 stabilized GFP-MTCL tumor cells into female mice through tail vein. The mice were sacrificed at different day after the tumor injection and multiple organs were dissected from the mice, i.e. lungs, liver, kidney, ovaries, great omentum and bone marrow. The whole amount sections were prepared from each of these organs and amounted onto fluorescent microscope for searching the GFP-MTCL tumor cells. At third day after tumor cell injection, the GFP MTCL tumor cells were found in every organ that we studied. Majority of these tumor cells were distributed throughout the organs as individuals or in 3-5 cell clusters. In the great omentum of one mouse, however, we found numerous micrometastatic foci sat nearby blood vessels (**figure 3 left**). At sixth day after the injection, most of the widely spread individual cells were disappeared; while in some of the organs, i.e. lungs, we found a few micrometastatic foci on the surface of lungs (**figure 3 right**).

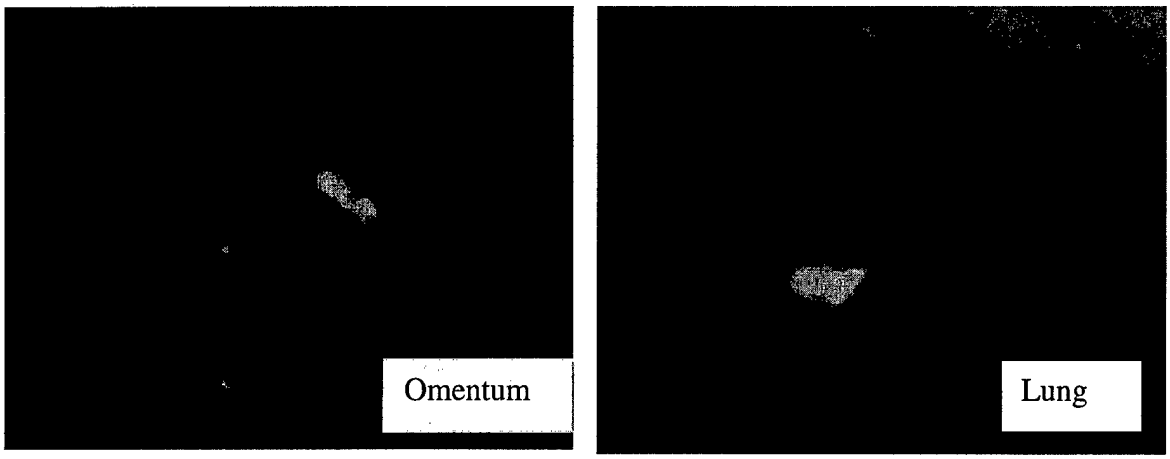


Figure 3.

Task 5- Elevate hormone levels in OVX mice prior to transplant with tumors using pellets with estradiol, progesterone or prolactin-> Resect tumors-> Follow up recurrence with lung metastasis. Ongoing data analysis.

Technical Objective #2: Cellular immune studies in intact and OVX tumor bearing, sex hormone repleted mice before surgical resection and four different times following surgical resection.

Task 6, 7- Resect tumors in ***intact and ovx*** mice at one of four fertility cycle stages-> Follow up cellular immune response assays (NK cell assays and perform T cell subset determinations) on day 14 (pre-op) and post-op days 17, 20, 23 and 26. We have conducted two experiments last year in which the tumors were resected from intact and ovx mice at one of four fertility cycle stages. We have accessed the cellular immune function of the tumor tissues, and we also have broadly analyzed the gene expression profiles in the tumor tissues (please see the following two abstracts).

(1). Fertility cycle coordinates molecular pathways responsible for cancer growth and spread

In order to better understand the molecular mechanisms responsible for the fertility cycle coordination of breast cancer growth and spread, we transplanted MPT4 mouse mammary tumor cells into the right hind legs of 83 and 18 ovx female mice and resected these tumors two weeks later, in an attempt to cure. The primary tumor tissues from these mice were frozen (half) or fixed (half) in 10% buffered formalin and embedded in paraffin blocks. After the tumor removal, about half of the mice developed a lethal locally recurrent tumor at the surgical site. Those which did not (35/83, 42.2% - normal female and 10/18, 55.56% -ovx) were subsequently sacrificed when approximately 5% of them had died from pathologically proven metastatic cancer to lung. Thorough autopsies determined whether metastases were or were not present in each mouse. Surgical cure was most frequent when cancers were resected from mice during estrus and least frequent when they were resected during diestrus ($p<0.01$). These resected tumors were used to create tissue microarrays to assay for proteins related to cellular immune function (NK, CD3, CD4, CD8, interferon gamma and TNF alpha*), tumor growth (Her-2/neu, cyclin E), DNA repair and apoptosis (BRCA-1, bcl-xs), sex hormone sensitivity (ER alpha, PR) and metastatic spread (cathepsin L, MMP9) by immunohistochemical staining. Bcl-xs (pro-apoptosis) and BRCA1 (a DNA repair factor) were each expressed most robustly

within tumors resected at estrus (100%, 100%), and was lowest in tumors resected at diestrus (38.5%, 69.2%). Conversely, the expression of cathepsin L peaked in tumors resected at diestrus (75.0%), and was weakest at estrus (28.6%). Her-2, ER, PR, MMp9 and cyclin E were expressed more or less equally throughout the estrous cycle. The results of the present study demonstrate that the molecular pathways responsible for tumor cell DNA repair, apoptosis, and the capacity to break down basement membrane are coordinated in breast cancer cells by the female fertility cycle. These dynamic changes of tumor cell protein expression may be responsible, in part, for the cycle's coordination of breast cancer growth and post-resection metastatic potential. *ongoing data analysis

(2). cDNA microarray assay and gene expression profiles in MTP tumors:

One of our recent investigations focused on the relevance of the sex hormonal milieus/ menstrual cycle stage to tumor metastatic spread. In a group of 26 mice, local subcutaneous MTP tumors were established at the tibia of mouse hind legs. These tumors were subsequently resected at early tumor growth stage by the hind leg amputation. Following resection of the tumor-bearing limb, mice were sacrificed 3-4 weeks later and the incidence of tumor recurrence, by identification of lung tumor nodules was evaluated. We demonstrated a 100% surgical cure frequency (with no lung metastasis) in mice when their tumors were resected during estrus phase *vs.* 28.5% cure rate in mice when their tumors were resected during the diestrus phase (P=0.0269). We have cryo- preserved their tumor samples and are now conducting cDNA microarray based gene expression analyses to define all possible genes, which are either turned on or turned off by changing the sex hormonal milieus/estrous cycle stages. To solve the problem of tumor cell heterogeneity, we have successfully established a protocol for laser microdissecting tumor cells from a frozen section. In our preliminary experiments, we have isolated fair quality of RNA from the microdissected tumor cells, which were proved by doing real time PCR amplification using known genes as targets, i.e. *Her2/neu, S16*. We have established protocols for labeling these RNA with Cye3 and Cye5 and protocols for doing the cDNA microarray chip hybridization. We compared the gene expression profile of one MTP tumor, which was dissected from a mouse at estrous stage and cured after the tumor removal, with that of another MTP tumor, which was dissected from another mouse at diestrous stage and died later from the lung metastases. As the figure shows, the diversities in gene expression profiles of these two tumors are obvious (**figure 4**).
To focus our research attention upon the genes that have been known relevant to tumor metastatic spread and immune function, we have started to construct our own oligo DNA microarray chip, which contains two hundreds of the genes that are interested.

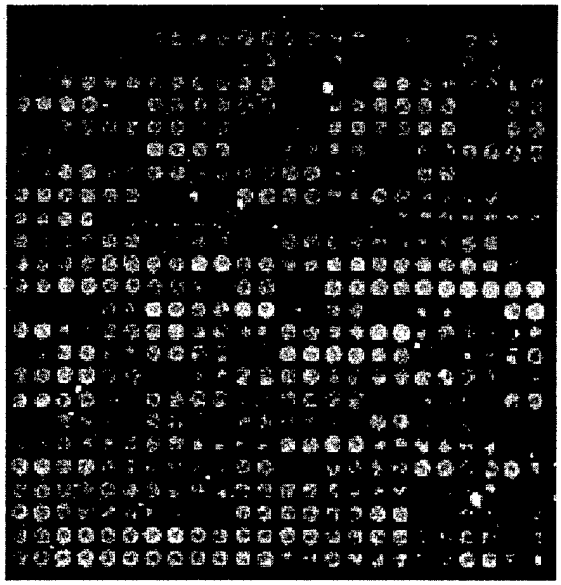


Figure 4.

Technical Objective #3: Determination of the effect of depleting NK and/or T suppressor cells and/or T helper cells upon the hormone dependence of post resection cancer spread.

Task 8- Perfect dose and schedule of antibody administration that ablates NK function (asialo GM-1) and depletes NK cell number, T helper cell (CD_4) and T suppressor cell (CD_8) numbers. Administer these antibodies prior to and following resection at each fertility cycle phase (P,E,M and D) and determine the effects of these immunocyte manipulations upon cure frequency and metastatic burden. ***Grant Transfer and No Cost Extension requested (Years 2 and 3) to accomplish these tasks.***

Task 9- Ongoing and interim data analysis with preparation of data for presentation at cancer meetings (AACR), USAMRMC conference and manuscript(s) preparation and submission. Interim data analysis with preparation of data for presentation at cancer meetings and USAMRMC conference. ***Grant Transfer and No Cost Extension requested (Years 2 and 3) to accomplish these tasks.***

Key research accomplishments:

- Recruitment of Kathy Bove PhD to work in this area (publication attached).
- Mentored peer reviewed funding of Dr Bove VA Merit type II award 2000-2003.
- Recruitment of Shaojin You PhD to work in this area (publication attached).
- Mentored peer reviewed funding of Dr You VA VISN & CDA 2002-2004.
- Recruitment of a PhD student Yin Xiong to work in this area.
- This work has stimulated 43 other retrospective clinical studies . most of which have confirmed our finding.
- The institutional and completion of the first prospective study confirming our results.
- We have established a cell line which shares this biology and can be manipulated genetically to determine its molecular mechanisms.

Reportable outcomes:

Publications:

1. Hagen A, **Hrushesky W.** Menstrual timing of breast cancer surgery. Am J Surg 1998;104:245-261.

2. **Hrushesky W,** Vyzula R, Wood P. Fertility maintenance and 5-fluorouracil timing within the mammalian fertility cycle. Reprod Toxicol 1999;13:413-420.

3. **Hrushesky W,** Lester B, Lannin D. Circadian coordination of cancer take and metastatic spread. Int J Can 1999;83:365-73.

4. **Hrushesky W**. Rhythmic Menstrual Cycle Modulation of Breast Cancer Biology. J Surg Oncol 2000; 74: 238-241.

5. Retsky M, Demicheli R, **Hrushesky, W.** Premenopausal Status Accelerates Relapse. *BREA* 65 (3), 217-224, February/March, 2001.

6. Retsky M, Demichelli R, **Hrushesky WJM**. Wounding from Biopsy and Breast Cancer Progression. Letter to *The Lancet,* Vol 357, No 9261, 31 Mar 01, page 1048.

7. Bove K, Lincoln D, Wood P, **Hrushesky WJM**. Fertility cycle influence on surgical breast cancer cure. Breast Cancer Research and Treatment 75:65-72, 2002.

Submitted for Publication:

1. Demicheli R, Retsky M, **Hrushesky WJM.** Menopausal status dependence of the early mortality reduction due to smaller tumors diagnosing (T1 vs. T2-T3): relevance to breast cancer screening.

2. Bove K, Wood P, Chambers A, **Hrushesky W**. Molecular Mediators of Angiogenesis are Modulated by the Fertility Cycle in Normal Mammary Tissue and in Mammary Tumor.

3. **Hrushesky W**. Improbability of the metastatic cascade. Perspectives in Biol and Med.

4. Shaojin You, Wei Li, Minoru Kobayashi, Yin Xiong, William Hrushesky, Patricia Wood. Creation of a Stable Breast Cancer Cell Line That Maintains Fertility Cycle Cancer Biology of the Parent Tumor.

Abstracts:

1. **Hrushesky W,** Wood P, Bove K. Mammalian fertility cycles modulate cancer growth and spread. 3rd Annual VA Oncology Cancer Symposium. San Antonio, TX, 1998.

2. Bove, K, Wood, PA, **Hrushesky W.** Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) expression in breast cancer varies with fertility cycle stage. Proc AACR 40: 1999.

3. Bove, K, Wood, P, **Hrushesky, W.** Molecular mediators of angiogenesis are modulated by the fertility cycle in breast cancer (abstract). Joint meeting of the eighth international conference of chronopharmacology and chronotherapeutics and the american association for medical chronobiology and chronotherapeutics, Williamsburg, VA, 1999.

4. **Hrushesky W,** Bove K, Wood P. Breast cancer resection within the menstrual cycle: risks, costs and benefits. 1st Milan Breast Cancer Conference. Milan, Italy, 1999.

5. Bove K, Wood P, **Hrushesky W.** Molecular mediators of angiogenesis are modulated by the fertility cycle in breast cancer. 4th annual va oncology cancer symposium. Washington, DC, 1999.

6. Wood, P, Lincoln, D, Bove, K, Clark, R, **Hrushesky W.** Tumor thymidylate synthase (TS) activity and mRNA vary rhythmically throughout the day. Proc AACR 2000.

7. Bove, K, Lincoln, D, Wood, P, **Hrushesky, W.** Reproductive cycle coordination of tumor growth and post-resection local recurrence of a low metastatic potential, gene marked, breast cell line. Proc AACR 2000.

8. **Hrusheksy W,** Bove K, Chambers A, Wood P. Reproductive cycle and tumor stage affect measures of cell proliferation and angiogenesis. In: Breast Cancer Research Program, Era of Hope; 2000 June 8-12; Atlanta, GA; 2000.

9. Wood P, Bove K, **Hrushesky W.** Meaningful and reproducible reproductive cycle modulation of cancer biology. In: Seventh Meeting Society for Research on Biological Rhythms; 2000; Amelia Island Plantation, Jacksonville, Florida; 2000.

10. Kobayshi M, You S, Wood P, Rich I, **Hrushesky WJM.** Prominent Circadian and Estrous Cycle Impact upon Tumor Biology. Eighth Meeting of the Society for Research on Biological Rhythms. Amelia Island Plantation and Conference Center, Jacksonville, Florida; May 22-26, 2002.

11. You S, Kobayashi M, Rich I, Musk P, Wood P, **Hrushesky WJM.** Tissue and cDNA Microarrays for High-Throughout Molecular Profiling of Cancer Chronobiology. Eighth Meeting of the Society for Research on Biological Rhythms. Amelia Island Plantation and Conference Center, Jacksonville, Florida; May 22-26, 2002.

12. **Hrushesky WJM,** You S, Kobayashi M., Wood P. Fertility Cycle Coordinates Molecular Pathways Responsible for Cancer Growth and Spread. Era of Hope 2002 DoD Breast Cancer Research Program Meeting, Orlando, Florida; September 25-28, 2002.

13. Wood P, You S, **Hrushesky WJM**. Fertility Cycle Coordinates Molecular Pathways Responsible for Cancer Growth and Spread. 7th Annual VA National Cancer Symposium, Alexandria, Virginia; October 2-4, 2002.

Conclusions:

Not only is cellular immune function coordinated by the estrous cycle but new blood vessel formation, tumor cell proliferation and apoptosis are each coordinated by this important biological rhythm. We conclude that the mechanisms of this biology are complex and interactive. We are continuing to unravel this clinically important puzzle.

References:

Please see the "reportable outcomes".

Breast Cancer Research and Treatment **75:** 65–72, 2002.

APPENDIX 1

Report

Fertility cycle influence on surgical breast cancer cure

Kathleen Bove[1], David W. Lincoln[1], Patricia A. Wood[2], and William J.M. Hrushesky[2]
[1]*Research Service, Stratton VA Medical Center, Albany, NY;* [2]*Research Service, Dorn VA Medical Center, Columbia, SC, USA*

Key words: breast cancer, estrous cycle, metastases, surgical cure

Summary

Cancer growth and spread is an intricate process dependent upon both tumor and host. This laboratory is interested in the role of the fertility cycle, specifically cyclic changes in steroid hormone levels, in tumor growth and metastases. Our previous studies, using a murine model, have documented that breast cancer growth rate and post-resection metastatic behavior each change reproducibly during the estrous cycle, and that post-resection cancer spread depends upon the time within the estrous cycle that an advanced transplanted cancer is resected. Twelve to thiry-two percent cure rates were seen in these studies. That early work described estrous cycle stages just prior and near to putative ovulation to be superior while those stages farther from ovulation were disadvantageous times for surgery. Data presented here confirm the role of the estrous cycle in post-resection metastatic spread. This current work validates vaginal smear determined estrous cycle stage with uterine weight. A primary, transplantable, mammary carcinoma, which metastasizes to the lungs, was resected for surgical cure in cycling C_3HeB/FeJ female mice at each fertility cycle stage. A group of oophorectomized (ovx) animals was also used. In two large, independent studies resecting much earlier stage cancers than in prior studies, a 96% surgical cure frequency was documented when the tumor is resected during estrus. The second best surgical cure rate is achieved when tumors are resected during metestrus (79% overall cure rate). Cure frequency in ovx animals is intermediate. These results further support a probable role for circulating E_2 and P_4 levels in modulating the metastatic process. We conclude that the timing of surgical resection within the estrous cycle affects the cancer's metastatic potential and that the optimal timing of resection may also depend to some extent upon the size (stage) of the resected cancer.

Introduction

Cancer growth and metastasis are influenced by many complicated and interrelated physiologic processes. These modulators of cancer virulence may reside primarily within the tumor itself (e.g., hormone receptors, tumor suppressor, and oncogene expression, production of autocrine growth factors and receptors, cell proliferation/apoptosis dysregulation). Others are characteristics residing primarily in the host (e.g., paracrine and endocrine factors, age, sex, fertility cycle stage, immune status, surgical stress and wounding). Different hormonal milieus associated with differences in sex, age, and fertility cycle stage, may alter tumor behavior either through direct effects upon tumor cells themselves and/or through their effects upon host dependent processes that then indirectly modulate tumor behavior. Prominent sex differences in the clinical cancer outcome following surgery and/or cytotoxic chemotherapy have been described [1]. Several retrospective studies have shown that there is a distinct 10-year survival advantage for premenopausal women who undergo surgical resection of their breast cancers during the luteal phase compared to the follicular phase of the menstrual cycle (reviewed in Hagen et al., 1998) [2]. We are particularly interested in the potential influence of reproductive hormones, such as estradiol (E_2) and progesterone (P_4), on the stress and wounding response induced by surgical resection, as reflected by the subsequent rate of post-resection metastatic spread.

Tumor metastasis is ultimately responsible for most cancer deaths [3]. Successful treatment occurs more often in tumors which have not metastasized at the time of diagnosis than in those which have already spread when the tumor is detected [4]. A better

understanding of the process of metastasis, and in this case its hormonal controls, would be useful in order to improve upon the survival of patients with cancer and increase the frequency of cure.

We investigated the influence of the estrous cycle on breast tumor surgical cure and metastatic spread to the lungs, using a primary, transplantable, mammary carcinoma, resected for surgical cure from young, sexually mature cycling C_3HeB/FeJ female mice at each of four fertility cycle stages. Oophorectomized (ovx) animals were also used to determine the effect of minimal E_2 and P_4 levels upon metastatic potential. Our prior work in this model system was done at a stage in tumor growth when an average 25% of resected mice were apparently cured [5]. That work demonstrated superior outcomes for mice resected during proestrus (32% cure) and estrus (25% cure), while the worst outcomes were experienced in mice resected during metestrus (12% cure) and diestrus (22% cure). The current studies were initiated at earlier stages of tumor growth in order to see if the estrous cycle dependence of cure was relevant at more curable stages of cancer growth, as is the clinical situation. In these studies we also measured uterine weights and an S-phase marker in order to validate the cycle stage of the mouse at the time of cancer resection.

Materials and methods

Animal and tumor model

Animals

All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee, Stratton VA Medical Center (Albany, NY). Two separate experiments were performed. The first included 112 and the second included 100 sexually mature, female C_3HeB/FeJ mice (Jackson Laboratories, Bar Harbor, ME), 8 weeks of age, housed four per cage, alongside singly housed male mice, to enhance estrous cycling as in our previous studies [6, 7]. Twenty animals were not included in the study as they either died during surgical procedures (oophorectomy, tumor resection), or never developed a tumor. All animals were kept on lighting schedules with 12 h light alternating with 12 h of dark with food and water freely available. Time of day (circadian time) is referenced to hours after light onset (HALO). In the second study, bilateral oophorectomy was performed at 10 weeks of age. Animals were allowed to recover for 2 weeks prior to tumor injection. Confirmation of oophorectomy was accomplished through serial vaginal diestrus cytology.

Tumor

The breast cancer primary tumor (B. Fisher, Univ Pittsburgh) originated spontaneously in a female C_3H mouse and was passed *in vivo* in C_3HeB/FeJ female mice [8]. This is an estrogen receptor (ER)-α, ER-β, and progesterone receptor positive tumor (Bove K, unpublished). Tumors were harvested under sterile conditions and tumor cell suspensions were made by gentle grinding of minced tumor pieces over a stainless steel mesh into Medium 199 (Gibco-BRL, Grand Island, NY). Tumor cells were inoculated subcutaneously at 2×10^4 viable cells in the right hind leg during the early activity phase (14 HALO) in both studies. Tumors were measured daily (length, width, height) by the same individual, using calipers. Tumors were excised from animals in one of four estrous stages and from oophorectomized animals, at an average size of 900 mm [3] by surgical removal of the tumor bearing right leg. In the first study, surgical tumor resection occurred at two different times of day (during the early activity phase (14 HALO) and in the early sleep phase (2 HALO)). Time of day at these two specific times of resection was found not to affect tumor metastatic potential, so in the second study tumors were resected only at 14 HALO. Animals were monitored daily for local tumor recurrence. Animals with local tumor recurrence at the site of resection (57% study 1, 45% study 2) were considered local surgical failures and were not included in further analyses for distant metastases. All animals were sacrificed (when 5% of the animals died from lung metastases) and autopsied for the presence or absence of lung metastatic lesions. In study 1 the number of visible lesions/lung were determined. In study 2 the number of visible lesions/lung and lung weights were recorded. Uteri were removed at vaginal cytology determined estrous cycle stages, weighed, and stored at −80°C.

Fertility phase determination

Daily vaginal smears were done using sterile saline washings, stained with Diff Quik (J.T. Baker, Phillipsburg, NJ) and were read by one individual using standard criteria [9]. Slides from each mouse were read in a daily sequence 24 h apart to determine the

progression of cycling and classify smears as proestrus (P), estrus (E), metestrus (M), or diestrus (D). Estrous cycle stage was determined daily, for 4 days prior to tumor inoculation and subsequently until the day of tumor resection to assess the precise estrous stage of tumor resection. Uterine wet weights were recorded, in a separate group of tumor bearing animals, to validate our estrous cycle stage classification ($n = 112$).

Reverse transcription-polymerase chain reaction

Uteri were rapidly collected, homogenized and total RNA recovered (TRIZOL, Gibco-BRL). First strand cDNA was generated from 1.0 μg of total RNA using SuperScript II reverse transcriptase (Gibco-BRL). Quantitative PCR was performed according to the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer, Foster City, CA) using ^{32}P-labeled dCTP. Oligonucleotide paired primers for mouse ribosomal protein S16 and histone 3.2 were purchased from Gibco-BRL. PCR samples were fractionated by electrophoresis on an 8% PAGE and quantitated by phosphorimage analysis (STORM 860, Molecular Dynamics, Sunnyvale, CA). The linear range of amplification was determined for each primer pair. Results are expressed as the ratio of the gene of interest to control gene for each sample (i.e., histone 3.2/S16). The RNA signal for histone 3.2/S16 did not vary statistically significantly throughout the cycle.

Statistical analyses

Parametric analyses

Numerical values for uterine weight were contrasted across the four estrous cycle phases, and also in the ovx state (second study) using one way ANOVA with SuperANOVA statistical software. Nonparametric analysis (Chi^2 analysis) was used to determine whether the proportion of animals cured across the cycle was randomly distributed using SPSS statistical software.

Results

Estrous cycle stage classification validation

To confirm our vaginal cytology-based estrous cycle stage classification we analyzed uterine wet weights and mRNA levels of a marker of uterine proliferation (histone H3.2), from groups of mice sacrificed at each estrous cycle stage (Figure 1). Uterine weight varies as a function of the estrous cycle. Uteri obtained from mice during the progesterone-estrogen rich cycle phase of proestrus and the estrogen-rich phase, estrus, are heaviest ($F = 3.59$, $p < 0.03$, Figure 1(A)). The cell proliferation/S-phase associated marker, mRNA for histone 3.2, also varies as a function of the estrous cycle paralleling wet weight and consistent with known changes in uterine cellular proliferation (Figure 1(B)).

Local tumor recurrence

Local tumor recurrence at the site of inadequate resection of the primary cancer presumably proceeds by a different set of cellular mechanisms than does hematogenous spread. Overall local tumor recurrence occurred in 51% of the mice. In neither study 1, nor study 2, were the proportions of local recurrence affected by the timing of resection within the estrous cycle (Figure 2(A): p,e,m,d; $X^2 = 0.53$, $p < 0.91$) (Figure 2(B): p,e,m,d; $X^2 = 4.2$, $p < 0.24$). Since the patterns of local recurrence were not identical, the combined data from these two studies demonstrate no clear cycle stage dependence (Figure 2(C): p,e,m,d; $X^2 = 3.7$, $p < 0.3$). Although statistical significance was not reached at the 0.05 level, it is interesting to note that the lowest local recurrence frequency occurred when mice were resected in the estrus phase of the cycle, the time within the cycle associated with the lowest post-resection hematogenous metastatic spread (*vide infra*). Time to local tumor recurrence does not vary as a function of the estrous cycle at resection (Study 1: $F = 1.9$, $p < 0.15$; Study 2: $F = 0.8$, $p < 0.53$ – data not shown).

Metastatic spread of tumors

In Study 1, the time of day of tumor resection, between the two circadian stages studied, did not affect the frequency of subsequent metastatic spread ($X^2 = 0.83$, $p < 0.36$). Similar results were obtained when animals were resected at either early sleep phase (2 HALO) or early activity phase of the circadian cycle (14 HALO) (Table 1).

The estrous cycle stage of resection did affect the frequency of metastatic spread in study 1. The greatest surgical cure frequency (100%) was found in animals whose tumors were resected during estrus (Figure 3(A)). Those animals with tumors resected during metestrus faired second best with 88.5% cure rates. Animals resected in either diestrus or proestrus

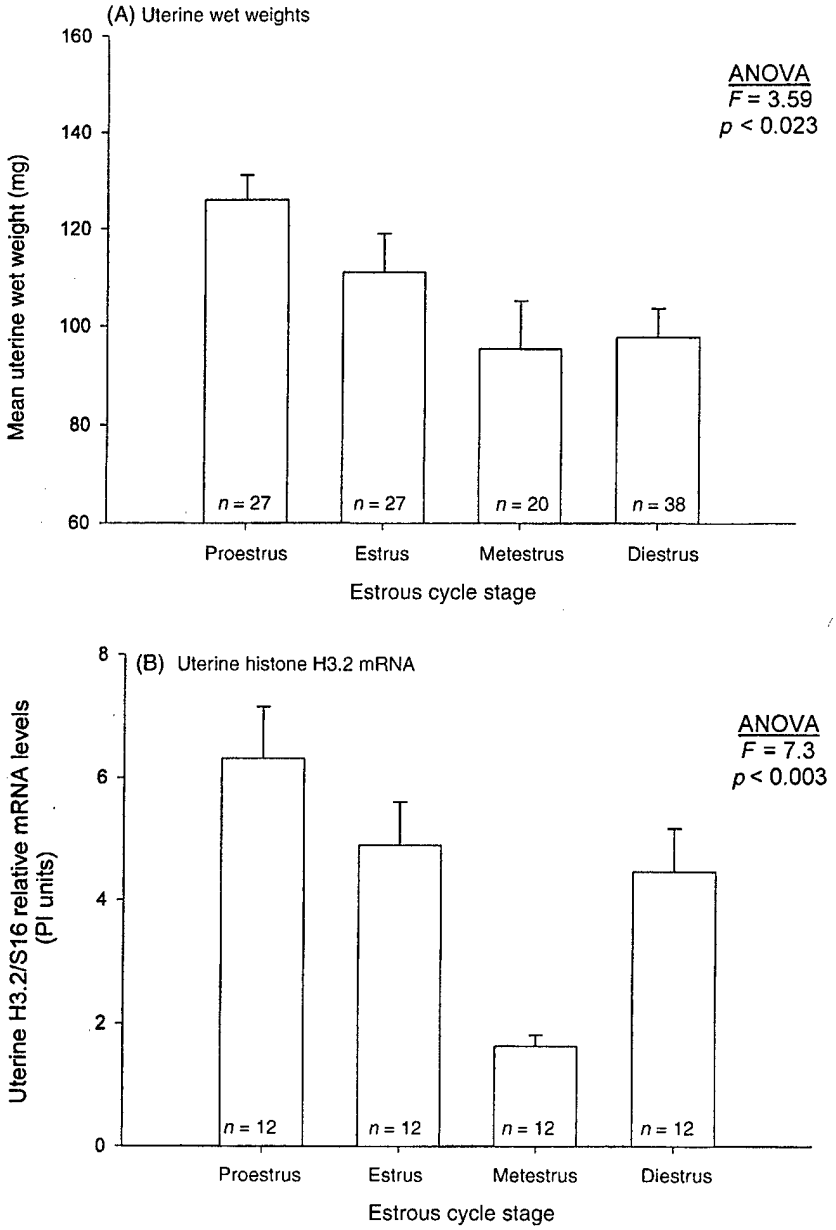


Figure 1. Confirmation of estrous cycle stage classification. (A) Uterine wet weights as a function of estrous cycle stage. (B) RT-PCR of uterine histone 3.2 mRNA levels as a function of estrous cycle stage. PI units = phosphorimage units.

had the lowest surgical cure rate (40 and 20%, respectively). The number of total lung metastases does not vary as a function of estrous cycle stage at resection ($F = 0.56$, $p < 0.58$ – data not shown). On average, tumor sizes did not vary as a function of estrous cycle at time of resection (data not shown; $F = 4.1$, $p < 0.1$).

Comparable estrous cycle specific tumor curabilities were obtained in the second study. There was a 93% surgical cure rate in those animals that had their tumors resected during estrus (Figure 3(B)). The second best surgical cure was found in animals whose tumors were resected during metestrus (67%). Sur-

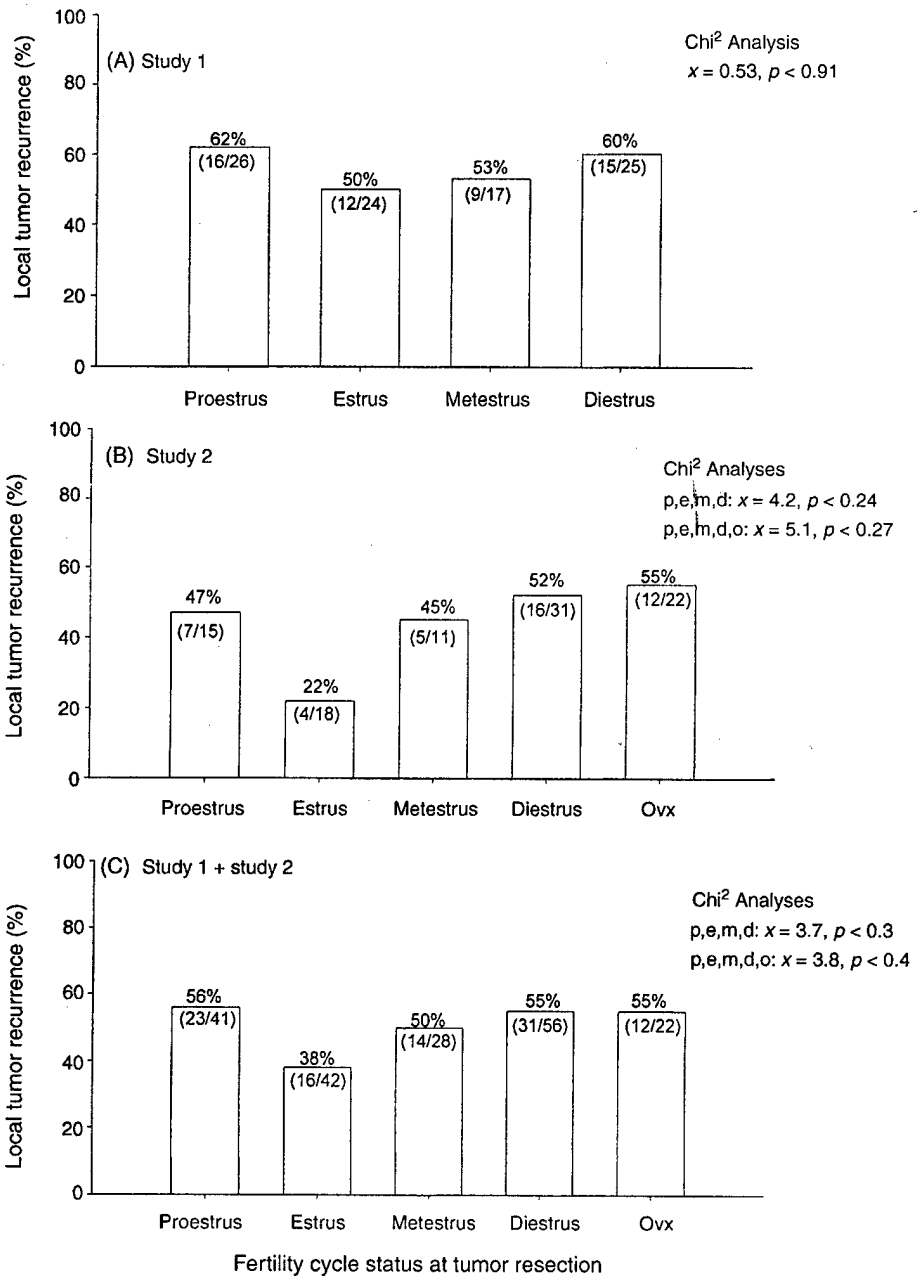


Figure 2. The effect of estrous cycle stage at time of resection on local tumor recurrence. Percentage of animals, from each estrous cycle stage, with locally recurring tumors. (A) Study 1. (B) Study 2. (C) Combined data from study 1 + study 2.

Table 1. (A) Time of day effect on fertility cycle-regulated surgical cure/study 1

Time of surgery	2 HALO	14 HALO
	Percentage of surgical cure	
Estrous stage at resection		
Proestrus	33% (1/3)	14%(1/7)
Estrus	100% (5/5)	100% (7/7)
Metestrus	86% (6/7)	100% (1/1)
Diestrus	0% (0/2)	50% (4/8)
	Chi^2, p	
	9.6, $p < 0.022$	11.4, $p < 0.01$

(B) Time of day effect on overall surgical cure/study 1

HALO at resection	Percentage of surgical cure
2 HALO	71% (12/17)
14 HAL0	50% (13/26)
	Chi^2, p
	0.83, $p < 0.36$

gical cure in animals who were resected during proestrus, diestrus or in ovx animals were similar (50, 47, 50%, respectively).

The combined data from these two studies are shown in Figure 3(C). Together these studies demonstrate that the fertility cycle stage at the time of tumor resection has a major effect upon surgical cure/metastatic spread (X^2: $F = 24.6$, $p < 0.0001$).

Discussion

These studies demonstrate that the spread of breast cancer through the blood stream to different sites is affected by the sex hormone milieu at the time of potentially curative surgical resection. The optimal timing for resection is during the 9–15 h long estrus [10], near, at or shortly after ovulation, a time of high and falling progesterone and shortly after the first proestrus peak in estrogen when it too is highest and falling toward basal levels [11, 12]. Concurrently, there are dramatic changes in pituitary hormone concentrations occurring. Circulating levels of the pulsatile lutenizing hormone (LH) rise rapidly and peak in late proestrus and immediately decline to basal levels by estrus [12]. Follicle stimulating hormone pulses peak during mid-proestrus (simultaneously with LH), begin to decline and then show a second series of peaks in early estrus [13]. Prolactin levels parallel LH levels, peaking in late proestrus and then rapidly declining to basal levels by estrus [13]. Such dramatic changes in the hormonal milieu in the brief span from late proestrus to mid-estrus apparently have an impact upon tumor physiology and the host-cancer balance. This salutory effect is most prominent in estrus and is largely maintained during metestrus when estrogen rises and falls rapidly for a second time, now in the absence of progesterone. Both hormones fall to their lowest levels in diestrus when metastases are most frequent. The final preovulatory estrous cycle phase, proestrus, is associated with rapid rises in both hormones and poor cure frequencies in these studies of relatively early stage breast cancers.

These data differ somewhat from those previously reported in this laboratory. In earlier studies, tumors were resected at a more advanced stage. In previous studies, on average, far fewer cures were achieved and the greatest surgical cure frequency was found in animals resected near or at estrus [6]. Ratajczak et al., classified animals into only two categories ('near estrus' and 'post estrus') while we have herein performed a more thorough, classic, 4 stage estrous reading [9]. The current studies also confirmed the identification of the vaginal cytology determined cycle phases by examining corresponding uterine wet weight and an independent marker of uterine cellular proliferation (Histone H3.2). Uterine wet weights are consistently higher in proestrus and estrus as a result

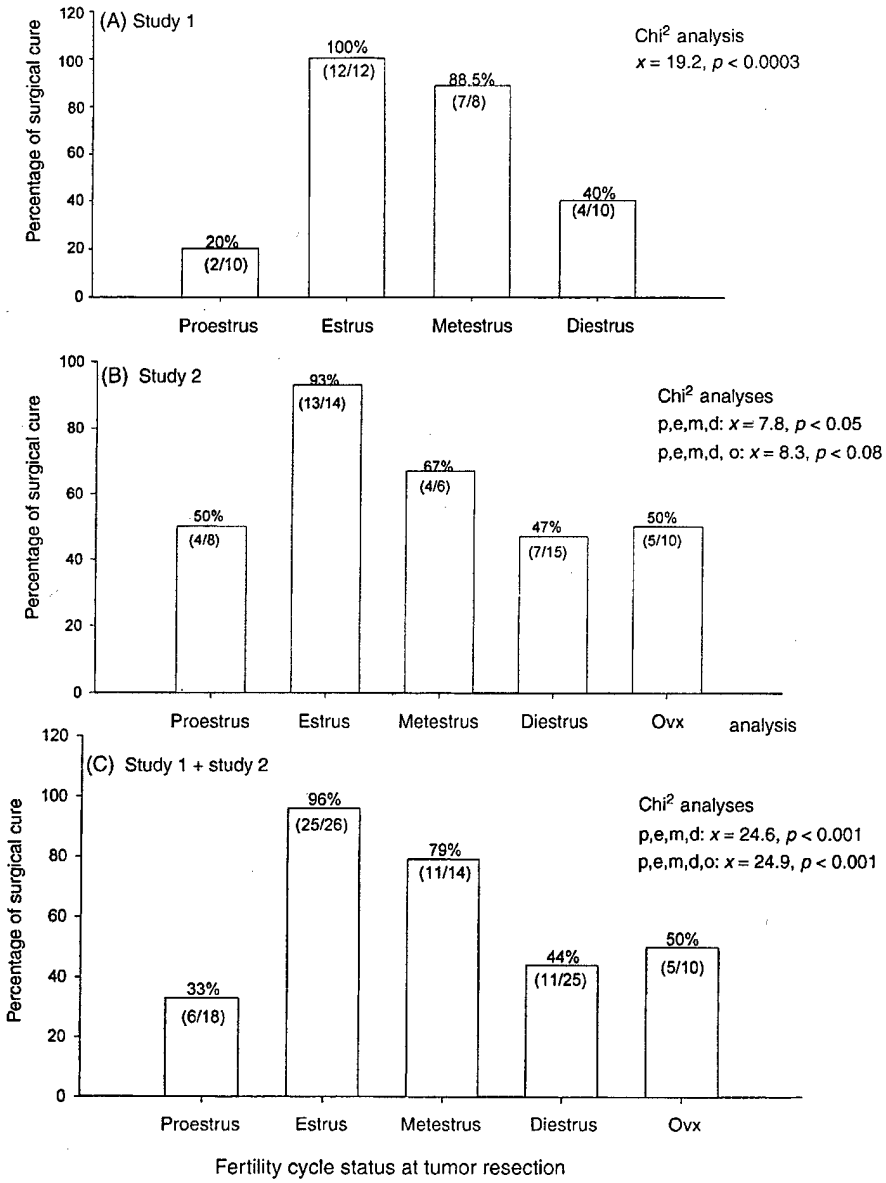


Figure 3. The effect of estrous cycle stage at tumor resection on surgical cure. Tumors were resected for cure from animals in each of the estrous stages. Percentage of surgical cure in these animals is determined by the absence of metastatic lesions in their lungs. (A) Study 1. (B) Study 2. (C) Combined data from study 1 + study 2.

of the estrogen-induced increases in hyperemia and water imbibition (reviewed in Clark and Markaverich, 1988) [14]. Estrogen also increases cellular proliferation in the uterus during these same estrous cycle phases [14]. Other work indicates that the host-cancer balance changes with the stage of tumor growth [15]. It is possible that the hormonal milieu which is more favorable for the cure of less advanced cancers differs from that optimal for cure of more advanced, less surgically curable cancers.

It is logical to speculate that the microenvironment at and near the time of estrus (characterized by high but declining P_4 levels) is responsible for alterations in the gene expression patterns of factors

controlling metastatic spread of this tumor. We have begun to analyze gene expression patterns in these tumors to determine if there is a 'metastatic' marker present which is either turned on or turned off by changes in sex steroid hormone levels during certain stages of the fertility cycle. Analogous clinical data exist using tumor samples obtained from premenopausal women with breast cancer, along with menstrual cycle information coupled with hormonal monitoring. Saad et al., examined mRNA levels of various genes whose expression has been associated with metastatic potential [16]. Expression levels of genes associated with tissue basement membrane degradation and metastatic spread, cathepsin L and MMP-9 and p53 are higher in human breast cancers resected during the follicular and periovulatory phases of the menstrual cycle compared to other phases of the cycle. These data show that metastatically relevant malignant propensities of human breast cancer change dramatically during each menstrual cycle [15].

In summary, data in this paper show that the metastatic process is reproducibly modulated by the timing of breast cancer resection within the mammalian fertility cycle. The optimal resection timing is that span at and shortly following ovulation associated with maximum fertility and the highest pulsatile levels of LH, FSH, prolactin and the high *albeit* falling levels of estrogen and progesterone. Therapeutic advances can be achieved in the mouse and in young women with breast cancer by timing the potentially curative tumor resection to this phase of the cycle. It remains to be seen whether pharmacological re-creation of a similar hormonal milieu to this cycle phase will enhance curability in cyling and/or postmenopausal mice or women.

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Creation of a Stable Breast Cancer Cell Line That Maintains Fertility Cycle Cancer Biology of the Parent Tumor

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Running title: **Establish a Breast Cancer Cell Line**

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Abstract

A mammary tumor cell line, designated MTCL, was successfully established from a mouse primary mammary tumor (MTP). The MTCL cells retain both estrogen receptors (ER) and progesterone receptors (PR) *in vitro*. *In vitro* exposure of MTCL cells to progesterone causes a decrease in the cellular ^{3}H-thymidine uptake, indicating an inhibition by progesterone on MTCL cellular DNA synthesis; while exposure of the cells to a high dose of estrogen (15 pg/ml) for 48 hours causes an increase of ^{3}H-thymidine uptake. We inoculated both MTP or MTCL tumor cells into normal cycling female C_3HeB/FeJ mice and demonstrated that the post resection metastatic recurrence of MTCL tumors, like the original MTP tumors, depends upon the time of tumor resection within the mouse estrous cycle stage. We demonstrated that both MTCL and MTP tumors have similar histological appearances but with significant differences in tumor necrosis and vascularity. Equivalent levels of sex hormonal receptors (ER α, ER β, and PR), epithelial growth hormonal receptors (Her2/neu, EGFR1), tumor suppressors (BRCA-1, P53), and cell apoptosis relevant protein (bcl-xl) were found in these *in vivo* tumors, except that the cyclin E protein was significantly higher in MTP tumor cells comparing to that in MTCL tumor cells. Our results indicate that MTCL cells retain many of biological features of the original MTP primary tumor cells, and to our knowledge, it is the first *in vitro* cell line that has been shown to maintain the unique estrous cycle dependence of *in vivo* cancer metastasis.

Key Words: Mammary neoplasma, surgery, fertility cycle, metastasis

Introduction

The cells of the human breast, as well as cancer cells arising within this organ, are profoundly affected by female sex hormones. Beatson (Beatson et al, 1896), more than one hundred years ago, demonstrated that removing the ovaries, which cyclically produce sex hormones (progesterone and estrogen), from young women with advanced breast cancer caused the cancer to shrink and in some cases disappear entirely. In the ensuing years, many of the biochemical and molecular connections between sex hormones and breast cancer progression have been better defined. Because of our interest in biologic rhythms, such as the estrous (in mice) / menstrual (in women) cycle, we did a series of tumor resection experiments in groups of cycling female mice (Bove et al, 2002; Ratajczak et al, 1988). We found that an operation designed to cure mice of mammary tumors did so two to three times more frequently if the surgery was done during the time of the cycle when both progesterone and estrogen were present in high concentrations. We also found that mammary tumor growth waxes and wanes during the mouse fertility cycle, with the highest tumor growth rate at the diestrus stage and the lowest growth rate at the estrus stage (Kobayashi et al, 2002). It is logical, therefore, to suspect that the cyclic changes in sex hormonal milieu in estrous /menstrual cycle stages are responsible for the obvious fertility cycle differences in tumor growth and metastatic potential following tumor resection.

Cell lines, established from human and rodent mammary tumors, are the most commonly used *in vitro* models for breast cancer research. Each of the cell lines has its own unique biologic features, which have facilitated cancer research in many ways. It is often challenging to establish a cancer cell line from a well-characterized primary tumor that retains its original biological features. Primary tumor cells in culture for long periods tend to lose the unique molecular characteristics of the parent cancer. For examples sex hormone receptors may be lost or altered. These molecular alternations may subsequently change tumor biologic behavior, i.e. enhance/diminish tumor growth, invasion and/or metastatic potential (Hambly et al, 1997). To study the influence of fertility cycle and sex hormone modulation of tumor surgical curability, it is best done in a syngenic host-tumor model where the immune system remains intact. We recently succeeded in establishing a new tumor cell line from a well-characterized mouse primary mammary tumor – MTP

that can be studied in vitro and in syngenic intact host in vivo. We have demonstrated that this new breast cancer cell line retains many of the in vitro and in vivo biologic features of the original MTP tumors including fertility cycle stage dependent metastatic potential. The availability of *in vitro* and *in vivo* tumor systems concurrently will accelerate exploration of the mechanisms by which the estrous/menstrual cycle stage at the time of cancer resection affects metastatic spread and breast cancer outcome.

Material and methods

Animals and housing: Female C3HeB/FeJ mice were purchased from Jackson Laboratory at 4-5 weeks of age and allowed to acclimate for two months until 12 weeks of age. Mice were housed (four per cage) in an environmentally controlled animal facility with 12 hours light alternating with 12 hours dark. All experiments were in compliance with the NIH Guide for Care and Use of Laboratory Animals and have been approved by the Institutional Animal Care and Use Committee (the VA Medical Center Animal Care and Use Committee).

Cell culture: In general, standard aseptic techniques of cell culture were employed. Cells were grown in tissue culture incubators with a 5% CO_2 atmosphere at 37^0C and passaged at confluence. Cells were cultured in RPMI-1640 medium containing 5-10% fetal bovine serum (FBS, GIBCO Invitrogen Co) and antibiotics (100 IU/mL penicillin G, and 25g/mL gentamicin). In experiments where cells were treated with sex hormones, phenol red free medium with 5% FBS was used. The experimental controls were treated in parallel by adding the same volume of vehicle to cell culture medium.

Establishment of mouse mammary tumor cell line (MTCL) *in vitro*: A piece of fresh sterile MTP primary tumor tissue was placed in ice-cold RPMI-1640 medium immediately after surgical resection from the mouse, and a single-cell suspension was prepared by forcing the tumor tissue through a metal screen. After centrifugation at 250x *g* for 10 minutes, the cell pellet was re-suspended in culture medium. The single cell suspension was seeded at a concentration of 2 x 10^6 viable cells in 60 mm culture dishes (Falcon, Becton Dickinson Inc., Franklin Lakes, NJ). The culture medium was first exchanged the next day, when cell attachment and spreading were observed, and then refreshed twice a week. The cultured cells were trypsinized and passed into new dishes when the cells reached confluence. The subcultures were continually maintained in the

above culture medium and eventually cryo-preserved, at the 50^{th} passage, in liquid nitrogen following the standard procedure for long-term storage.

MTCL cell proliferation kinetics: The doubling time of the MTCL cell population at 50^{-th} passage was determined by daily cell numeration. On day 0, $1x10^5$ viable MTCL cells were seeded in three 60 mm dishes, which had grid lines on the bottom. Cells in the selected areas of each dish were counted under converted microscope daily, and the average cell number of three areas from each of three dishes was calculated. The tumor cell proliferation curve was plotted, and the cell doubling time was calculated based on the curve fitting equation (CA-Cricket Graph III).

Response to sex hormones by ^3H thymidine uptake:

To test the responsiveness of the tumor cells to sex hormones, MTCL cells were incubated with different concentrations of progesterone (Sigma) and estradiol (Sigma). The effect of these hormones on tumor cell DNA synthesis was determined by H^3 thymidine uptake assay performed in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ). The cells were seeded at a concentration of $2x10^4$ / well and incubated for 24 hours in phenol red free cell culture medium. The medium was then replaced by 1 ml fresh medium containing different concentrations of progesterone (30 ng/ml, 60 ng/ml and 90 ng/ml) or estradiol (5 pg/ml, 10 pg/ml and 15 pg/ml) or mixture of progesterone (60 ng/ml) and estrodiol (10 pg/ml), and incubated for 2, 4, 6, 12, 24, 48 hours. Each of these hormonal exposures was performed in sextuplicate. The range of concentrations of progesterone and estrogen were chosen as reference to the physiological concentrations of sex hormones in mice (Bergman et al. 1992; Michael 1976). The concentrations of progesterone and estrogen at the boundary phase of the proestrus and estrus stage in mice have been reported to be 60 ng/ml and 10.8 pg/ml, respectively. One hour prior to cell harvesting, 0.5 μCi of methyl-^3H thymidine (Amersham Pharmacia Biotech, Inc.) was then added to the wells and incubated for an additional hour. The medium was removed, and cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed with cell lysis buffer. The radioactivity in the lysate was counted with a liquid scintillation counter (LSC-5000, Aloka, Japan).

Mice oophorectomy: Female mice were anesthetized with an intraperitoneal injection of Ketamine (75mg/kg), acepromazine (1 mg/kg), and xylazine (10 mg/kg). A

small incision was made on one lateral side of the body, and the glistening white fat pad, which included the ovary, was pulled out. A hemostat was attached at approximately 5 mm below the uterus end, and the distal tissues were cut to remove the ovary. The surgical end was tied just beneath the hemostat, and the skin wound was then closed with a metal clip. The same procedures were performed on the other side of the body. Mice were allowed to recover from the surgery for two to three weeks. Daily vaginal smears were performed using sterile saline washings and stained with Diff Quik (J.T. Baker, NJ). An experienced pathologist read the vaginal smears using standard criteria (Allen et al, 1922) to determine whether or not the mouse's estrous cycle was ablated.

Tumor cell inoculation: Single-cell suspension was prepared from MTP primary tumors and MTCL cell subcultures. The cell viability was determined with Trypan blue (Sigma). The viable tumor cells ($2x10^4$) in 50ul basic RPMI medium without serum were inoculated subcutaneously on the flank of the mice for tumor growth study or on the right hind leg of mice for surgical metastatic study using a 0.5 ml syringe with a #28G1/2 needle.

Tumor size measurement and tumor growth study: Tumor-bearing mice were observed daily after the tumor cell inoculation and the tumor size was measured at the same time of a day by the same individual using callipers (length x width x height) until euthanasia at later stages of tumor growth. The tumor size-doubling times were calculated at the exponential growth periods of both MTP and MTCL tumors (the linear ranges of the tumor growth curves) based on the curve fitting equations (CA-Cricket Graph III).

Surgical tumor resection and metastasis study: From the second day after tumor inoculation, daily vaginal smears were performed and the mouse's estrous cycle stage was determined. Tumors, once they became measurable, were measured daily by the same individual and were resected by hind leg amputation in one of the four estrous cycle stages at an average tumor size of 600 mm^3 (11-12 days after tumor inoculation). Mice were then followed daily for three to four weeks. Mice were excluded from the analysis of curability if they were found with local recurrent tumor at the surgical wounding site. The remaining animals were continually followed and sacrificed when 5% of the mice died and autopsied. To document the presence or absence of metastatic foci, both lungs

were fixed in the Bouin's fixative (PolySciences. Inc) overnight. The white tumor metastatic nodules were examined and counted under a dissecting microscope (Fisher Scientics). Other sites (organs) of metastases were not grossly detected.

Preparation of tumor tissue array: Tumor tissues resected from mice in the tumor growth study were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. One hematoxylin and eosin stained section was prepared from each tumor specimen and examined by a pathologist to select vital and representative areas. This section was then aliened with the donor tumor tissue block for tissue array sampling. A tissue array instrument (Beecher Instruments, Silver Spring, MD) was used to sample and transfer the tissue cores into a pre-drilled hole on a recipient paraffin block. For each tumor block, triplicate tissue cores in 0.6 mm diameter were taken and arrayed side by side in the recipient block. Multiple 5 μm sections were then cut from the tissue array block, and the sections were mounted on the positive charged glass slides (SurgiPath).

Immunocytochemical/immunohistochemical staining procedures: For immunocytochemical analyses, MTCL cells were seeded in chamber slides (LAB-TEK II chamber slides, Nalge Nunc International) at a concentration of 1 x 10^5 cells/ml and cultured until 80% confluence. Cells were then washed with 1x phosphate-buffered saline (PBS) for 5 minutes and fixed for 15 minutes at -20°C with cold methanol.

For immunohistochemical analyses, the tissue array sections, after deparaffinization and hydration, were heated in microwave at 700 W in sodium citrate buffer (0.1 mol/l, pH6.0) 7 minutes twice. The slides for both immunostainings were then washed in PBS (pH7.2) 5 min two times. The tissue endogenous peroxidase activity was blocked by 3% H_2O_2 in PBS for 15 minutes. Slides were incubated with normal goat serum for 1 hour at room temperature. The properly diluted primary antibodies were amounted on the sections and incubated at 4^0C overnight. The secondary antibodies and AB complex (ABC staining kit, Santa Cruz) were applied and incubated for 30 min at room temperature respectively. Between the incubations, slides were rinsed three times (5 min/each) in PBS. The color was developed by 3,3'-diaminobenzidine tetrahydrochloride (DAB), and the sections were finally counterstained with Harris' hematoxylin. The negative control staining was performed without the primary antibody incubation. The rabbit polyclonal antibodies against estrogen receptor (ER α, 1:400, ER β, 1:200),

progesterone receptor (PR, reactive with both A&B subtypes, 1:400), her2/neu (1:400), EGFR1 (1:100), BRCA1(1:400), P53(1:200), cyclin E (1:100), bcl-xl(1:200) and CD31 (PECAM-1, 1:200) were purchased from Santa Cruz company. The sections were viewed using a Zeiss microscope (Germany), and images were captured using a SPOT digital camera (Diagnostic Instruments, Inc.).

Quantitative analyses of immunostaining signal in tumor cells: The tumor tissue array sections that were stained with the above antibodies were used for these quantitative analyses. The digital images from each of the tumor tissue cores were analyzed using SigmaScan Pro4 (SPSS Inc. Chicago, IL). Two areas, which were representative and consisted of only viable tumor cells, were selected from each tissue core. The objectives of measurement in each of these areas were defined and selected by an optimal pre-set intensity. The area and average intensity of the objectives were then measured, and total intensity was calculated as $Obj_{intensity}$ = average intensity x area. The mean of average intensity of all objectives from one tumor tissue core ($M_{intensity}$) was further calculated. The negative control slides were also measured in order to estimate the background stain intensity ($B_{intensity}$). We then modified the $M_{intensity}$ by subtracting the average background staining and the final formula for calculating the $M_{intensity}$ was: $M'_{intensity} = (\log 255 - \log M_{intensity}) - (\log 255 - \log B_{intensity})$ (Wells et al, 1992; You et al, 1996). The immunostaining signal intensities ($M'_{intensity}$) of the triplicate tissue cores from the same MTP or MTCL tumors were then averaged.

Tumor blood vessel count: Tumor tissue array sections were stained with CD31 antibody (1:100). The CD 31 positive stained tumor blood vessels were quantitatively evaluated. Images of the triplicate tissue cores from same tumor were captured and saved in files. Numbers of the tumor blood vessels in each of the triplicate tissue core were then counted. Average number of tumor blood vessels per area in each tumor was then calculated.

Statistical analysis: For each numerical value, such as the number of cultured tumor cells, number of tumor blood vessels, and tumor sizes, mean and standard deviation were calculated (standard errors were used in graphs). Comparison between two groups was done by *t* – test, and comparison between the means was done by One-way ANOVA with significance at $p<0.05$.

Results

Establishment of MTCL mammary tumor cell line *in vitro*

MTP tumor cells ($2x10^4$) were inoculated subcutaneously on the flank of a 3 month-old female C3HeB/FeJ mouse. Two weeks later, the tumor was resected, and a single cell suspension was prepared from the most vital part of the tumor. These tumor cells were then seeded in a 60 mm culture dish with RPMI culture medium. The culture medium was replaced on the second day of culture, when some of the cells had adhered to the bottom of cell culture dish. The shapes of the cells at this point were highly heterogeneous. After the first replacement, the medium in the culture dish was refreshed twice a week. The cultured cells, when they reached confluence, were trypsinized and passaged into new dishes. At the 50^{th} cell passage, the cell population was quite uniform. An epithelial-like tumor cell population (mammary tumor cell line, MTCL) became predominant. These epithelial tumor cells were polygonal in shape and varied in size, as are typical of malignant epithelial cells (figure 1). Although other cells, i.e. fibroblast cells (long spindle in shape) and small mono-nucleated cells (spheroid in shape) were observed at earlier stages of the culture, the number of these non-tumor cells decreased during successive cell passages, and very few fibroblast- like cells were found between the malignant epithelial cells at the 50^{th} passage. In order to estimate the proliferative rate of MTCL cells, we seeded equal numbers of MTCL tumor cells into triplicate dishes. We counted the cell numbers in selected areas daily. MTCL cells had a typical lag phase at the beginning of the culture and then grew exponentially after a day or two (figure 2). The cell doubling time at the linear range of the tumor cell proliferation curve was estimated to be 41 hours.

Expression of ER and PR proteins and response to sex hormones *in vitro*

Expression of sex hormone receptors, ER and PR, is a major biochemical characteristic of the original MTP mammary tumor cells. To determine if MTCL tumor cells retained expression of ER and PR, we cultured the cells in chamber slides and then stained the cultured tumor cells with anti-ER α, ER β, and PR antibodies immunocytochemically. Figure 3 show that most of the MTCL cells express ER α, ER

β and PR at 50th passage of the culture. These receptors are mainly found within the cell nucleus yet some of them are seen in the cytoplasm.

To evaluate the responsiveness of MTCL cells to sex hormones, we exposed the cells *in vitro* to different concentrations of progesterone (30 ng/ml, 60 ng/ml and 90 ng/ml) and estradiol (5 pg/ml, 10 pg/ml and 15 pg/ml) for 2, 4, 6, 12, 24, and 48 hours, and then determined the ^{3}H thymidine uptake to assess whether hormone exposure affected the tumor cellular DNA synthesis. Exposing MTCL cells to progesterone alone diminished cell capacity to take up ^{3}H thymidine (figure 4a). The ^{3}H thymidine uptake in progesterone exposed MTCL cells was significantly lower when compared with that of the vehicle exposed cells ($P < 0.01$). Conversely, exposing MTCL cells to a higher dose of estradiol (15 pg/ml) for 48 hours increased the rate of ^{3}H thymidine uptake (P= 0.045), although there were no changes in ^{3}H thymidine uptake with lower doses of estrogen exposures (figure 4b), (5, 10 pg/ml, P>0.05). We also exposed MTCL tumor cells to combination of progesterone (60 ng/ml) and estrdiol (10 pg/ml) for different times (2, 4, 6, 12, 24, and 48 hours). Similar to the case of exposing MTCL cells to progesterone alone, the combination of progesterone (60 ng/ml) and estrodiol (10 pg/ml) significantly decreased the rates of MTCL cell ^{3}H thymidine take up, indicating that the effect of progesterone on MTCL cellular ^{3}H thymidine incorperation was dominant (figure 4c).

***In vivo* tumor growth**

We inoculated 2x10^4 MTCL syngenic tumor cells subcutaneously on the flank of five ovx mice to test their tumorigenicity. As a parallel control, the same number of viable MTP original primary tumor cells was inoculated in another group of five ovx mice. We monitored the tumor growth daily for more than three weeks and sacrificed the mice at later stages of tumor growth. About 10 days after MTCL tumor cell inoculation, a palpable tumor was first found in three of the five mice. Two days later, a measurable tumor was formed in the other two mice. The average time for initial MTCL tumor appearance was 11 days (figure 6), four-days later than the average initial appearance of the original MTP tumors. After the initial appearance, however, MTCL tumor growth was faster than MTP tumor growth. The MTCL tumor size *in vivo* doubling time at the linear growth phase was about 31 hours, 10.8 hours shorter than that of MTP tumors (41.8 hours). At late stages of tumor growth, MTP tumor-bearing mice began to show

apparent body fat loss, slowed motion and ruffled hair appearance at which point they were euthanized. However, at similar tumor sizes, mice with MTCL tumors demonstrated normal eating, grooming and activity without apparent loss of body fat. All five of the MTCL tumor-bearing mice were physically well even though they were bearing bigger tumors.

Fertility cycle dependent surgical curability of MTP and MTCL tumors

The fertility cycle stage at primary tumor resection, as we have previously reported (Bove et al, 2002; Ratajczak et al, 1988), affects the MTP tumor distant metastatic recurrence rate. In order to compare the metastatic potential of MTCL tumors with that of original MTP tumors, we inoculated MTP or MTCL tumor cells into the right hind leg of two groups of normal cycling mice (34 mice in one and 42 mice in another). Both tumor and leg were resected when the tumor grew to an average size of 600 mm^3 (11-12 days after the tumor inoculation), and the tumor-bearing mice were observed for additional 3-4 weeks at which time they were euthanized to screen for lung metastases. In both of these tumor models, a fixed rate of local tumor recurrence was seen. We found that 23.5% of mice with MTP tumor and 31% of mice with MTCL tumors developed early local recurrence post resection. The local tumor recurrent rates demonstrated no association with the time of tumor resection within mouse fertility stages (MTP tumor, P=0.554; MTCL tumor, P=0.215). The mice without local recurrent tumors were continually followed, and sacrificed and autopsied later to score for the lung metastases. We demonstrated that 42.3% of mice with resected MTP tumors and 42.8% of mice with resected MTCL tumors developed lethal lung metastases. Excluding local recurrences, surgical cure rates were 28.6% and 35.3% when the MTP and MTCL tumor resection surgeries were performed within the diestrus stage, while 100% cure rates were found in both groups of the mice when the MTP and MTCL tumor resection surgeries were performed within the estrus stage (figure 5). The surgical cure rates of MTCL and MTP tumors were significantly associated with the surgical time within mouse estrous cycle stages (P=0.044, P=0.027) with nearly identical patterns in both tumor types. This biologic behavior is identical to that previously reported (Bove et al, 2002; Ratajczak et al, 1988).

Comparative histology of the MTP and MTCL tumors

Tumor tissues from the growth study from ovx mice were immediately evaluated grossly after they were dissected from the mice. The MTCL tumors appeared as encapsulated subcutaneous tumors with little invasion of underlying tissues. On gross inspection, instead of extensive necrosis, a typical feature of MTP tumors, MTCL tumors demonstrate only small patches of necrosis. These findings were supported by microscopic comparison of the H&E stained tumor sections (figure 7). The vital parts of both tumors, however, have similar light microscopic characteristics; both tumors consist of typical undifferentiated malignant epithelial cells.

Comparative *in vivo* breast cancer protein expression

Each of the five MTP and MTCL tumor tissues were fixed in formalin and embedded in paraffin blocks. Sample H&E slides were inspected and viable areas of predominant tumor cells were selected for more detailed studies. A tissue array block was created by biopsy of each tumor block at these microscopically optimal areas. Thirty tissue cores (three from each tumor sample) were arrayed side-by-side. Sections of this tissue array were cut and prepared for immunohistochemical assessment (IHC) of key tumor proteins. The expressions of all the selected target proteins in all tumor tissues were first assessed under the microscope and then quantitatively measured by digital image analysis. Sex hormone receptors (ER α, ER β, and PR) were expressed at equivalent levels in both MTP and MTCL tumors. Proteins related to epithelial growth hormonal receptors (Her2/neu, EGFR1), tumor suppression (BRCA-1, P53), and cell apoptosis (bcl-xl) were also expressed at similar levels in both tumors (Table 1). The signal intensity of the cyclin E immunostain, however, was significantly higher in MTP tumor cells than that in MTCL tumor cells. This immunostaining difference was also obvious under microscopic observation.

Tumor blood vessels in MTP and MTCL tumors

Because of large differences seen in tumor necrosis in these two tumors types, tumor angiogenesis in these tumors was quantitatively evaluated by staining tissue array sections with a polyclonal anti-CD 31 antibody, previously shown to allow the quantitative assessment of tumor blood vessels (Bevilacqua et al, 1995; Laforga et al, 2000). This staining demonstrated vascular endothelial cells and outlined the shapes of tumor blood vessels, which appeared in round or ovoid shapes (figure 8). In some of the

tumors, the blood vessels were extremely dilated, but in other cases, they were very narrow so that blood vessels appeared as endothelial cell cords. Tumor cells around the blood vessels appeared more robustly viable than those farther from the vessels; when necrosis occurred, it was always far from these CD31 positive areas. The total number of tumor blood vessels in each of the tumor cores was counted and averaged with the triplicate core specimens from the same tumor paraffin block since each core was of identical size and shape. MTCL tumors were significantly more vascularized than MTP tumors. The average number of blood vessels in MTCL tumors was 5.4 +/- 1.6 /core; while that in MTP tumors was 3.4 +/- 1.2 /core (P=0.0343). Tumor cells (both types of tumors) as well as other types of the normal interstitial cells adjacent to blood vessels expressed little or no CD 31 protein.

Discussion

Bernard Fisher's compelling work demonstrating the importance of host factors in determining whether lethal metastases develop was done with the parent MTP murine mammary tumor in the late 1950's (Fisher et al, 1959, 1968 b). Dr. Fisher observed and documented that tumor dormancy and spread was interrupted by surgery, resulting in post surgical metastatic cancer growth. Subsequent work of Pollock and Lotzova demonstrated transient yet profound cellular immunoparalysis in both mice and patients following surgery (Pollock et al, 1987a; Pollock et al, 1987b). We have investigated the inter-relationship among host, surgery, tumor and the fertility cycle. Our experiments with Fisher's breast cancer model demonstrate that the timing of potentially curative cancer resection within the fertility cycle determines the metastatic potential of that cancer and the ultimate curability of that mouse (Bove et al, 2002; Ratajczak et al, 1988).

The levels of sex hormones dynamically change during the female fertility cycle and affect the cellular proliferation in both the nulliparous murine mammary gland (Fata et al, 2001) and breast cancer (Badwe et al, 1995; Saad et al, 1998). In the normal murine mammary gland, Fata demonstrated specific morphological and cellular changes in the breast during each estrous cycle and defined their relationship to rhythmic physiological changes in steroid hormone concentration. He found that the mammary epithelial cell proliferation and apoptosis correlated better with progesterone than 17β-estradiol serum

concentrations. He proposed that the cyclical turnover of epithelial cells within adult mammary tissue is a sum of spatial and functional coordination of sex hormonal and matrix regulatory factors, e.g. the matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMPs). In human breast cancer, several studies measuring tumor growth and metastasis relevant gene expression (Badwe et al, 1995; Balsari et al, 1999; Saad et al, 1998) have correlated lower metastatic potential and favorable outcome with high progesterone levels, which occur in the early luteal phase of each menstrual cycle. Therefore, our demonstration of the responsiveness of MTCL tumor cells to progesterone is a reasonable step toward determining whether this newly established cell line remains useful for the chronobiologic study of breast cancer. The effect of progesterone on cell proliferation and apoptosis of human and mouse breast cancer *in vitro* has been controversial. Moore et al (Moore et al, 2000) and Ory et al (Ory et al, 2001) have reported that progesterone has an anti-apoptotic and/or pro-proliferative effect *in vitro* on PR-rich human breast cancer cells (TD47). Exposing TD47 tumor cells to progestins induced up-regulation of the anti-apoptotic protein bcl-xl expression. Formby et al (Formby and Wiley, 1999; Formby et al, 1998) and Gompel et al (Gompel et al, 2000) have, however, reported pro-apoptotic effects. Formby et al demonstrated a maximal 90% inhibition of cell proliferation with T47-D breast cancer cells (PR+) after exposure to 10 uM progesterone for 72 hours, while control MDA-231 cancer cells (PR-) were unresponsive to the progesterone exposure. We have demonstrated that progesterone obviously inhibits MTCL tumor cell DNA synthesis. This result is consistent with the results of Formby et al (Formby and Wiley, 1999; Formby et al, 1998) and Gompel et al (Gompel et al, 2000) and differs with those of Moore et al (Moore et al, 2000) and Ory et al (Ory et al, 2001).

One of the most interesting features of the primary MTP tumor model is the dependence of tumor metastatic spread post resection upon estrous cycle stage of that resection. To test if the newly established MTCL tumor cells retain this unique biologic feature, we inoculated MTP and MTCL tumor cells in separate groups of mice and resected their tumor for cure. We measured local tumor recurrence and lung metastatic spread and found that the estrous cycle dependence of tumor metastatic potential post resection was identical for both tumors. Tumor resection during estrus stage resulted in

the highest curability in both types of tumors, while resection during diestrus resulted in the lowest cancer curability. The idea of resecting breast cancer at defined menstrual/estrous cycle stage to improve outcome originally arose from Ratajczak's study using MTP tumor model (Ratajczak et al, 1988), which then stimulated many clinical studies. Since Hrushesky published the first clinical study in 1989 (Hrushesky et al, 1989), many well designed clinical studies involving thousands of breast cancer patients have shown that disease free or overall survival is reduced following surgery in the follicular phase compared with the luteal phase (Hagen and Hrushesky, 1998), while some of other studies found no correlation between timing of surgery in relation to the menstrual cycle and prognosis of premenopausal patients with breast cancer (Kroman et al, 1994; Nomura et al, 1999). Recent works by Bove et al (Bove et al, 2002) and Vantygham et al (Vantyghem et al, 2003) further confirmed this concept. In two large independent studies, Bove et al, using the same strain of mice and same tumor model as Ratajczak did, demonstrated 100% and 93% surgical cure rates when the tumor was resected during estrus stage versus 40% and 47% surgical cure rates during diestrus stage. In another study, Vantygham and colleagues delivered B16F10 melanoma cells into C57BL/B6 mouse circulation through tail vein injection at two different estrous cycle stages, the proestrus and metestrus stage. As expected, there was a large metastatic burden in the lung at 24 days after tumor cell injection, although the number and size of lung metastases did not differ between two injection stages. Unexpectedly, however, they found dramatic differences in extrapulmonary metastases between the different stages of injection. A total of 31.6% of mice injected in metestrus stage had ovarian metastases, while none of mice injected during proestrus stage had ovarian metastasis. They proposed that the fertility cycle timing of surgery may be more broadly applicable than to just breast cancer.

Subcutaneous tumor growth in the ovx mice was monitored daily and the growth kinetics of MTCL tumor *in vivo* was found to be somewhat different from that of MTP tumor. MTCL tumor had a longer average time to initial appearance compared to those of the original MTP tumors. There are several possible explanations for the slow initial growth of MTCL tumors. First, MTP tumor is a primary tumor line, which is usually considered a heterozygous tumor containing multiple subpopulations with different

growth potential. MTCL tumor cell line may be derived from one of the subpopulations with slower growth potential. Secondly, MTP tumor cell suspension contains a number of interstitial cells, such as fibroblasts, endothelial cells, macrophages, and lymphocytes. These cells are not only the passive witnesses of the tumor proliferation cascade but also may modulate tumor establishment and growth *in vivo*. Through secretion of their soluble (cytokines) and insoluble molecules (extra cellular matrix), interstitial cells mutually modulate tumor growth *in vivo* (Duffy et al, 2000; Hirtenlehne et al, 2002). Thirdly, the dose of injected tumor cells is another factor that may affect the initial tumor appearance, as described by Hrushesky (Hrushesky et al, 1999). To avoid injecting large number of necrotic MTP tumor cells and make a valid comparison with MTCL tumor cells, we made tumor cell suspension from the most vital part of MTP tumor, and injected the same number of viable MTP and MTCL tumor cells into mice. We, therefore, believe that the tumor initial appearances truly reflect their different growth potential.

Long term *in vitro* culture exerts selective pressure on the cultured tumor cells (Hambly et al, 1997). This pressure may change or alter the molecular pathways within the tumor cells. We compared the molecular fingerprints of the newly established MTCL derived tumors with those of the original MTP tumors. Eight of nine proteins, such as sex hormone receptors (ER α, ER β and PR) as well as proteins of several important epithelial growth factor receptors (Her2/neu, EGFR1), tumor suppressor genes (P53, BRCA1) and the gene that is relevant to cell apoptosis (bcl-xl) are all expressed equally in both parent and cell line derived tumors. This largely similar protein expression profile may account for, at least in part, the comparable metastatic potential of MTP and MTCL tumors. Cyclin E is the only protein that is expressed differently in these two types of tumors. The average intensity of cyclin E immunostaining in MTP tumors is much higher than that in MTCL tumors, indicating the higher expression level of this cell proliferation relevant protein in MTP tumors. Considering the obvious necrosis in late stage of MTP tumors and over expressed cyclin E protein, we assume that MTP tumor cells have faster tumor turnover rate than MTCL tumor cells. At early tumor growth stages, MTP tumor expresses more cell proliferation relevant proteins, i.e. cyclin E, resulting in faster tumor growth and earlier initial tumor appearance. At later stages of MTP tumor growth, however, tumor necrosis becomes apparent, which slows the speed of tumor size

increase. On the contrary, MTCL tumor does not show apparent necrosis since the tumor has a high density of tumor blood vessels.

In summary, we have established a new mammary tumor cell line (MTCL) from a well biologic defined mouse primary mammary tumor, which can be stored both in vitro and in vitro. This cell line expresses both ER and PR proteins and responds markedly to progesterone exposures *in vitro*. In comparison with the parent tumors, MTCL tumors demonstrated very similar molecular expression profiles, histological features, and metastatic potential and response to the fertility cycle *in vivo*. Availability of this tumor cell line, with its unique *in vivo* biological characteristics, will enable chronobiologists to construct many studies to determine the relationship of sex hormone exposure and tumor cell proliferation and/or apoptosis, and further investigate the interaction between estrous/menstrual cycle stages and tumor growth and metastatic spread.

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Table 1. Comparative in vivo protein expression in MTP and MTCL tumors as assessed by immunohistochemical staining and digital imaging quantitation

		Sex hormone receptors			Epithelial receptors		Tumor suppressors		Proliferation/ apoptosis	
		ER α	ER β	PR	*neu	EGFR1	P53	BRCA1	Cyc.E	Bcl-xl
MTCL	OD	0.26	0.22	0.21	0.21	0.20	0.19	0.20	0.12	0.21
	SE	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.01
MTP	OD	0.30	0.26	0.20	0.23	0.24	0.22	0.24	0.18**	0.23
	SE	0.02	0.02	0.01	0.02	0.03	0.01	0.03	0.01	0.02

- * Her2/neu
- **P value less than 0.01 for MTCL compared to MTP.
- OD=mean optical density (see material and methods), SE=standard error.
- Values are based upon a log scale.

Figure Legends

Figure 1. MTCL cell morphology *in vitro*. At the 50^{th} passage of cell culture, MTCL mammary tumor cells became the predominate cell population with triangular or polygonal cell shapes (arrows), the typical features of epithelial cells. (x40 phase objective lens).

Figure 2. *In vitro* growth kinetics of MTCL mammary tumor cells. MTCL cells were seeded in triplicate dishes and cell number was quantitated in selected areas daily. The cultured MTCL cells showed a slow initial increase in cell number and then proliferated exponentially. The cell population doubling time at the linear range of the growth curve is 41 hours, calculated based upon the curve fitting equation, $Y_{d1\text{-}d4}=116.4\ X_{d1\text{-}d4} - 134.7$. Values are means +/- standard errors.

Figure 3. *In vitro* sex hormone receptor expression in MTCL tumor cells. MTCL tumor cells were cultured in chambered glass slides and stained immunocytochemically with ER alpha, ER beta, and PR antibodies. Most of the MTCL cells are positively stained with these antibodies. The positive signals are mainly found in the nucleus (darker brown) and some are found in cytoplasma (lighter brown). In negative control slides (figure 3_{neg}), there is no positive staining. The cell nuclei are in blue. (x40 light objective lens).

Figure 4, *In vitro* H^3 thymidine incorporation. Dose and time course of H^3 thymidine uptake of MTCL cells in response to progesterone (a), estrogen (b) and progesterone plus estrogen (c). To compare the capacity of ^3H thymidine up take among different exposure times, we normalized each of the ^3H- thymidine radioactivity from sex hormone treated samples as the ratio to background radioactivity from simultaneous control cells. Progesterone (P) was used at 30, 60, and 90 ng/ml and estrogen (E) at 5, 10, and 15 pg/ml. Values are means +/- standard errors.

Figure 5. surgical cure rates with primary tumor resection performed at different estrous cycle stages (proestrus, estrus, metestrus, and diestrus) for MTP and MTCL tumors. Numbers within the bars are number of cured mice /total mice.

Figure 6. Tumor growth kinetics *in vivo*. MTP or MTCL mammary tumor cells were inoculated into oophorectomized mice (n=5/group) and tumor sizes were measured daily. The average initial time to appearance of MTCL tumor is 11 days, 4 days longer than that of MTP tumors (7 days). After tumor initial appearance, however, MTCL tumors grew faster than MTP tumors did. The exponential phase of tumor growth (the linear ranges of the growth curves - day7 to day 13 for MTP tumor, day 15 to day20 for MTCL tumor) was chosen for curve fitting analyses, and tumor-doubling times were calculated based on following equations: $Y_{MTP}=574\ X_{MTP} - 4642.6$, $Y_{MTCL}= 773\ X_{MTCL} - 11258.3$. The tumor doubling time of MTCL tumor is about 31 hours, 10.8 hours shorter than that of MTP tumors (41.8 hours). Values are means +/- standard errors.

Figure 7. Histological features of MTP and MTCL tumors. Sections were prepared from MTP and MTCL tumor blocks and stained with H&E. MTP tumors show interdispersed patches of necrosis (white arrow) with smaller areas of viable tumor tissues (blue arrow); while MTCL tumors show much less necrosis (large arrow) and more numerous blood vessels (small arrows). The cancer cells in both tumors appear as undifferentiated malignant epithelial cells (x10 light objective lens).

Figure 8. Tumor angiogenesis in MTP and MTCL tumors. Sections were prepared from MTP and MTCL tissue array block and stained with anti-CD31 antibody. The tumor blood vessels are shown clearly between tumor cells with varies of shapes, i.e. ovoid lumens (figure 8a) or very narrow and thin capillaries (figure 8b) (x40 light objective lens), and are more numerous in MTCL tumors than those in MTP tumors.

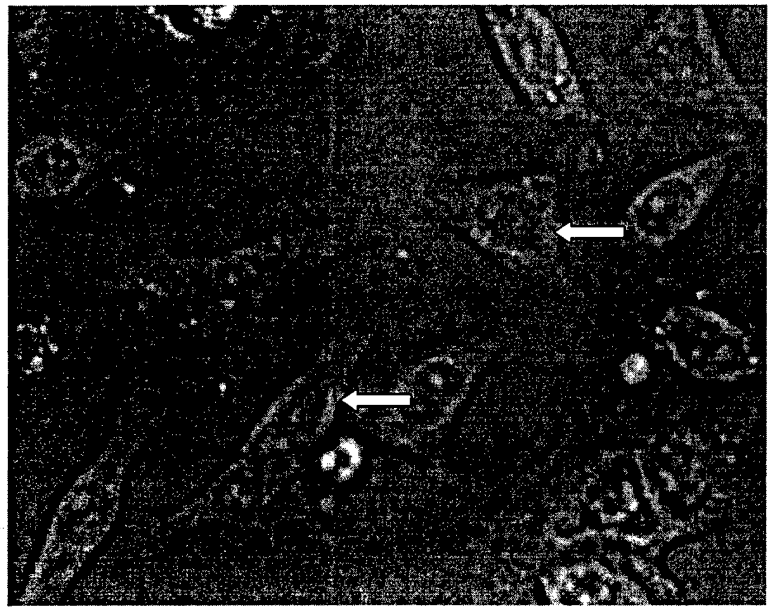

Figure 1.

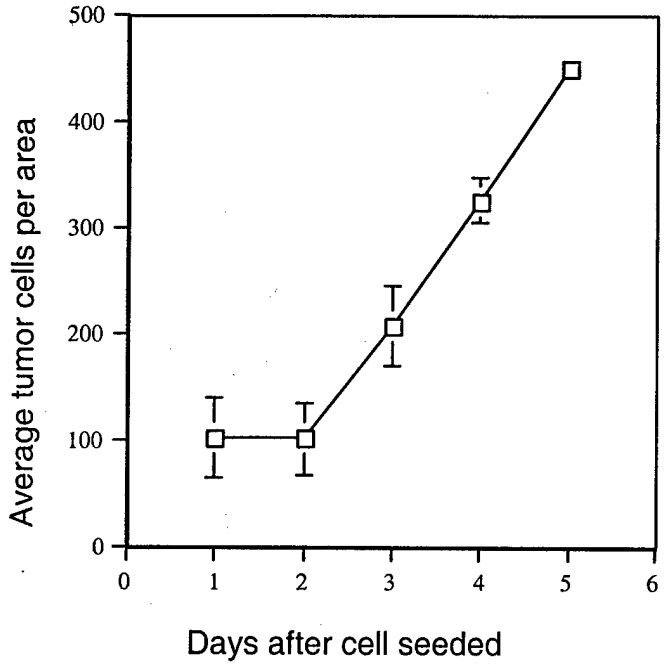
500
400
300
200
100
0
Average tumor cells per area
0
1
2
3
4
5
6
Days after cell seeded

Figure 2.

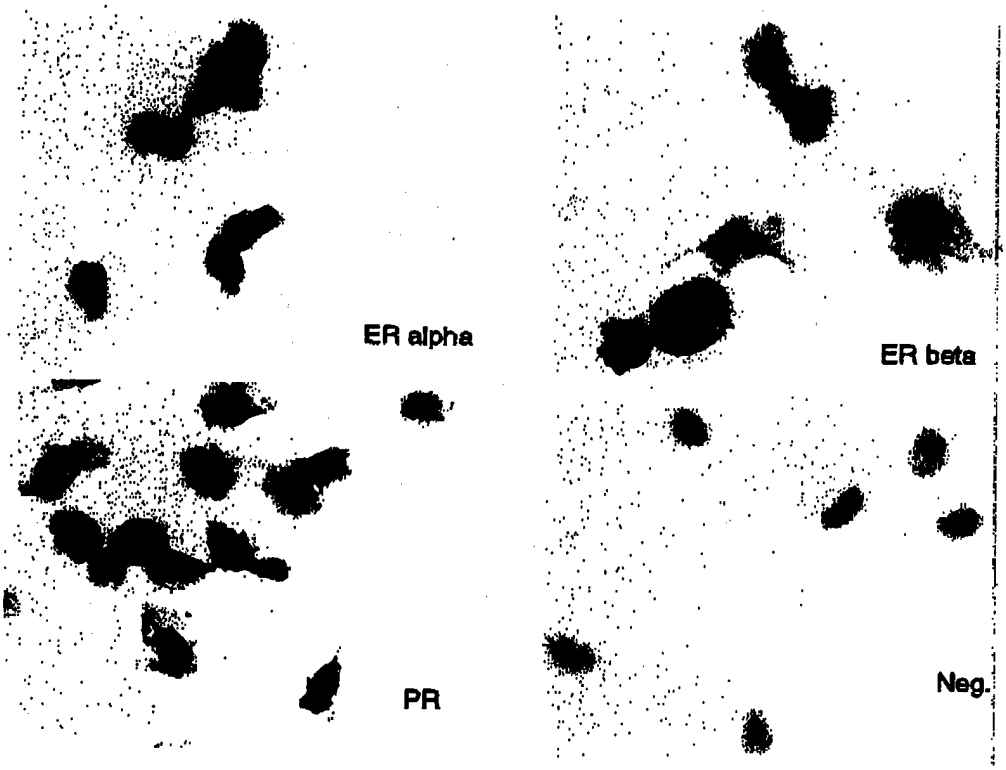

Figure 3

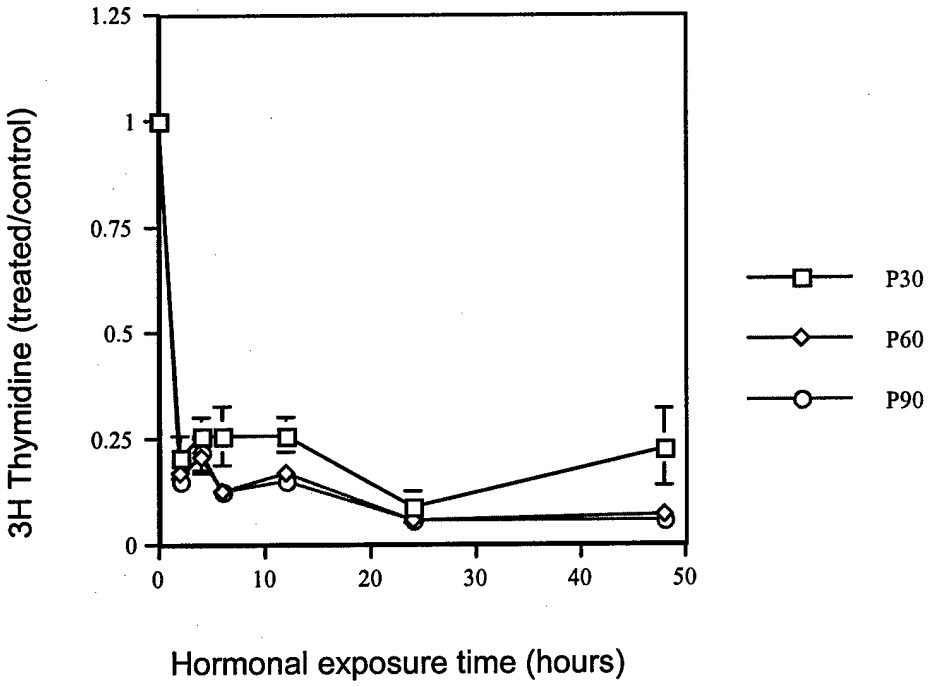

figure 4a

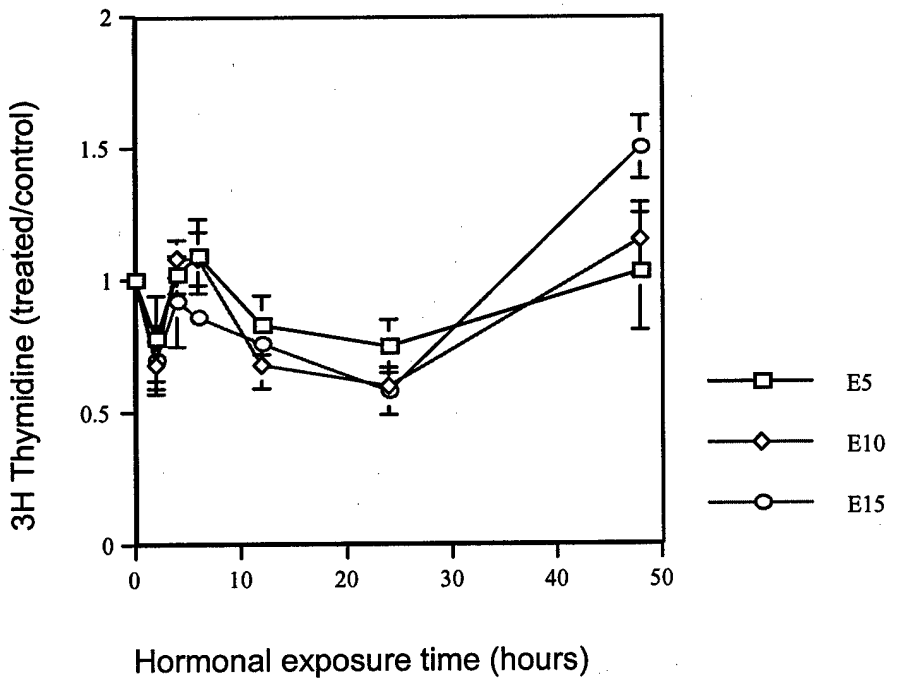

figure 4b

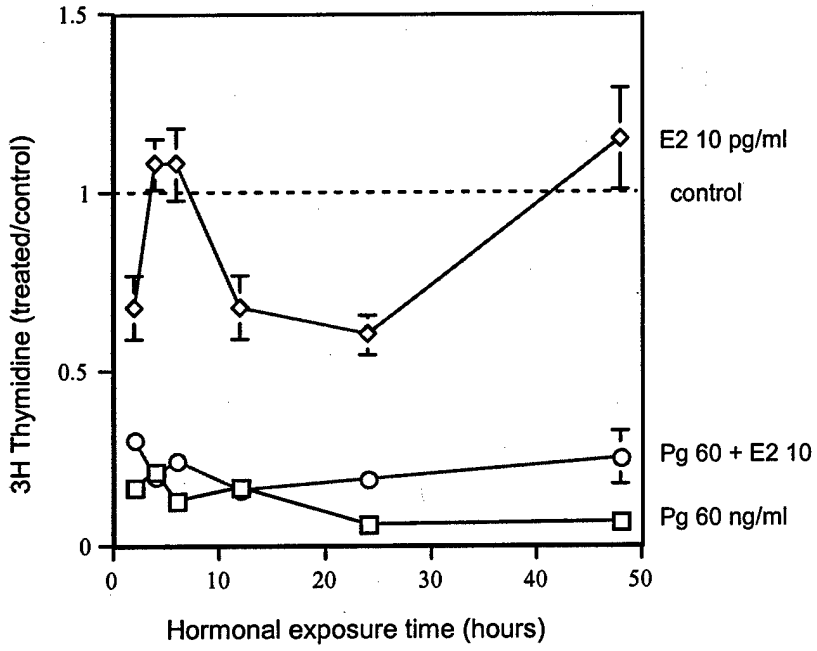

figure 4c

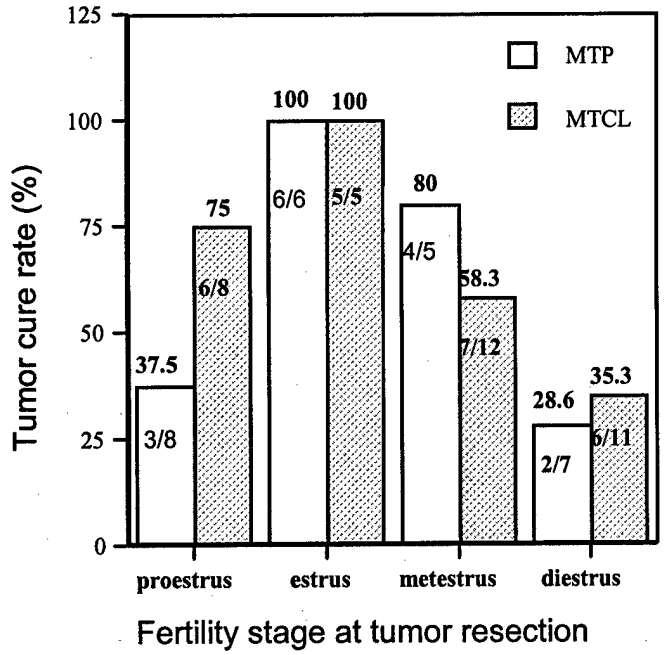

figure 5

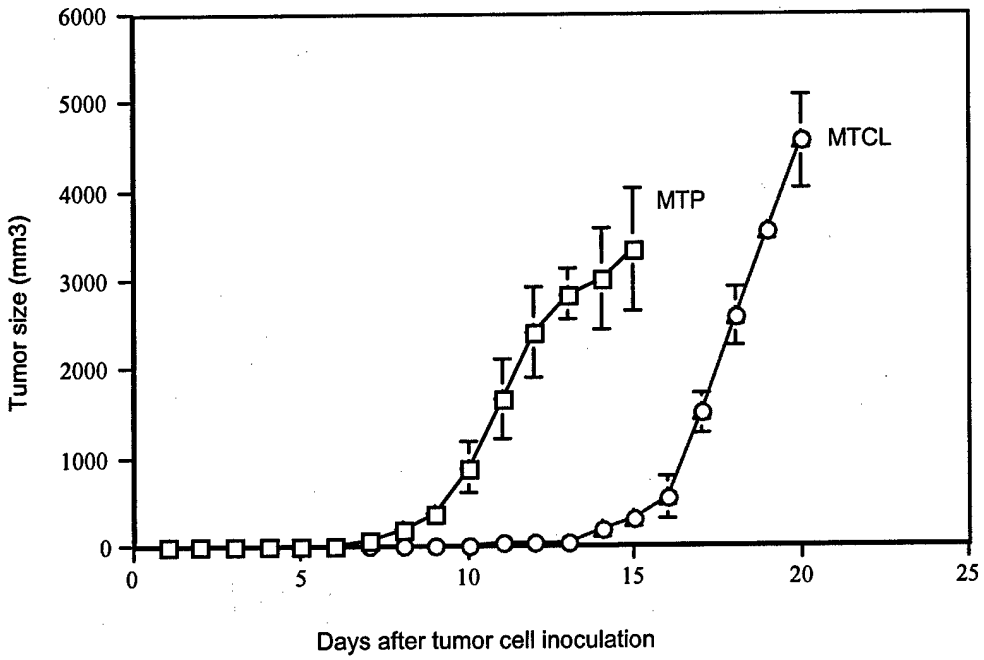
6000
5000
4000
3000
2000
1000
0
Tumor size (mm3)
MTP
MTCL
0
5
10
15
20
25
Days after tumor cell inoculation

figure 6

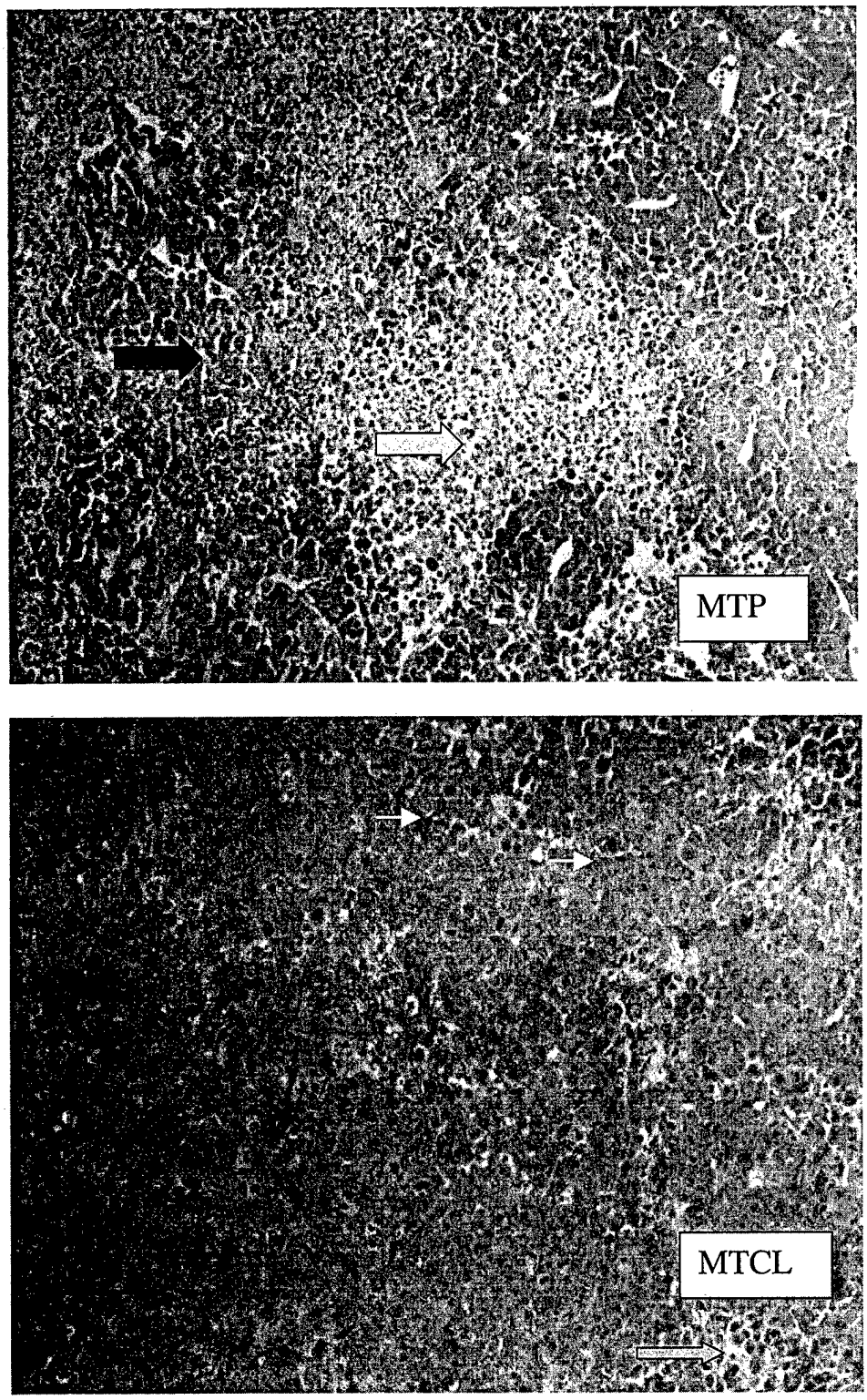

figure 7

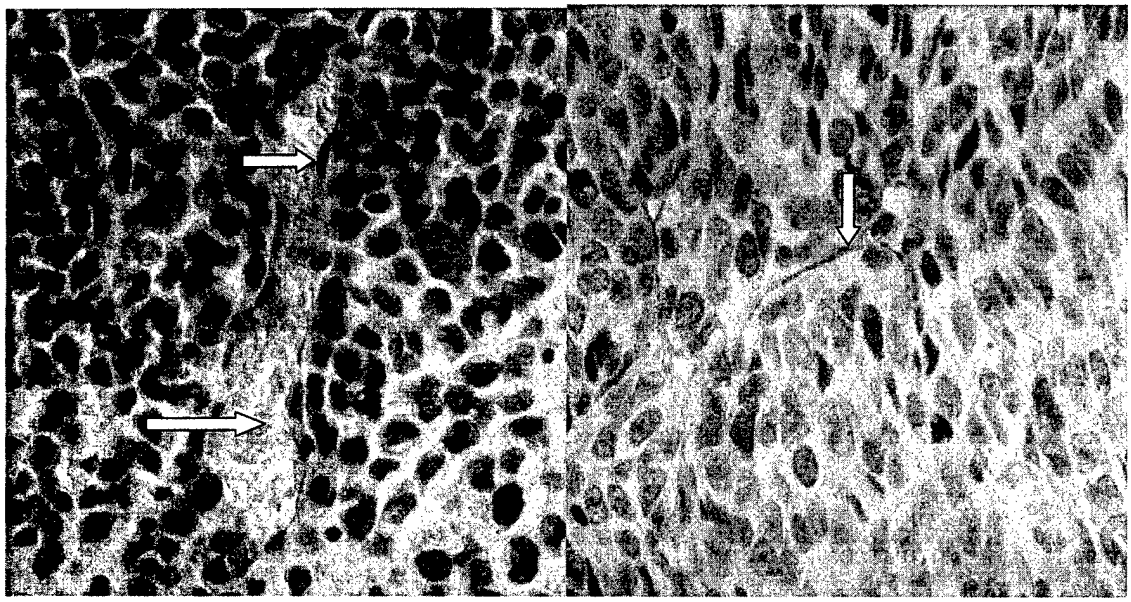

Figure 8.